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ENDEMIC TRICHINELLOSIS – EXPERIMENTAL AND EPIDEMIOLOGICAL STUDIES

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Academic dissertation

to be presented, with the permission of
the Faculty of Veterinary Medicine, University of Helsinki,
for public criticism in Walter Auditorium, Faculty of Veterinary Medicine,
Agnes Sjöbergin katu 2, Helsinki, on June 17th 2005 at 12 noon.

Helsinki 2005

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ISBN 952-91-8846-3 (paperback)
ISBN 952-10-2515-8 (PDF)
Yliopistopaino
Helsinki, 2005

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1. ABSTRACT

Trichinellosis is endemic in Finland. To evaluate the characteristics of the *Trichinella* parasite, epidemiological and experimental studies comparing domestic trichinellosis occurring among production animals with sylvatic trichinellosis in wildlife were conducted.

The prevalence of sylvatic trichinellosis in Finland was shown to vary by region, being higher in the southern regions than in the north. It was highest (70%) among lynxes in the southwest. In domestic animals, the dominating species was *T. spiralis. Trichinella nativa* was detected only in sylvatic hosts and was the predominant species among them. *Trichinella pseudospiralis* and *Trichinella britovi* were detected for the first time in Finland, and *T. pseudospiralis* for the first time in northern Europe. *Trichinella pseudospiralis* was detected both in synanthropic hosts that live close to human habitation, *i.e.* rats, and in sylvatic hosts. *Trichinella britovi* was detected only in mixed infections with other *Trichinella* species. The raccoon dog, the only host species to be infected by all four *Trichinella* species, also carried the most intense infections. An outbreak of trichinellosis was described on a wild boar farm. A likely source of the infection was a previous rat invasion in the area due to the closing of a nearby dump site.

The properties and applicability of molecular techniques RAPD-PCR and multiplex PCR were compared in identification of *Trichinella* species for epidemiological purposes. RAPD-PCR was very sensitive to the physical condition of the DNA analyzed, but multiplex PCR gave clear bands even when parasite DNA was not well conserved. Thus, multiplex PCR proved to be the superior method. Both methods did, however, yield good overall agreement in identification of *Trichinella* species.

Herbivore animals have been a source of human infections in several countries. The Finnish half-tamed reindeer was studied as a potential host for *Trichinella.* Reindeer were experimentally infected with the common parasite species of the domestic cycle (*T. spiralis)* as well as with the sylvatic and northern parasite species (*T. nativa)*. All inoculated animals seroconverted, but those inoculated with *T. spiralis* had more intense infection in their muscles. Exposed reindeer may therefore serve as a host for *T. spiralis*.

The persistence of *T. spiralis* in different environments was studied to simulate the infection routes from field to farm. Infected rat carcasses were incubated in silage, grained barley, and propionic acid-preserved feed, as well as under simulated pasture conditions. The parasites remained infective for two weeks in all environments and for up to four weeks in the propionic acid-preserved feed. Thus, contaminated feed may transmit infective rat carrion or other material to farm animals.

The traditional classification to domestic and sylvatic cycles may not be very useful in Finland. Special characteristics of the epidemiological situation in Finland include sporadic domestic pig trichinellosis (*T. spiralis*), presence of four *Trichinella* species in the same regions, presence of *T. spiralis* in sylvatic wildlife, presence of *T. pseudospiralis* in many host species, and mixed infections of several *Trichinella* species.

2. ABBREVIATIONS

ANOVA	One-way analysis of variance
BC	before Christ
CPK	creatine phosphokinase
CI	confidence interval
den	denier (unit of thickness of thread)
DNA	deoxyribonucleic acid
EC	European Commission
EEC	European Economic Community
EELA	National Veterinary and Food Research Institute
e.g.	*exempli gratia* (for example)
ELISA	enzyme-linked immunosorbent assay
ES	excretory-secretory
ESV	expansion segment V
et al.	*et alia* (and others)
EU	European Union
FAO	Food and Agriculture Organization
ICT	International Commission on Trichinellosis
i.e.	*id est* (that is)
IFAT	immunofluorescence antibody test
IgA	immunoglobulin isotype A
IgG	immunoglobulin isotype G
IgM	immunoglobulin isotype M
IIF	indirect immunofluorescence
ISS	Istituto Superiore di Sanità
ITRC	International Trichinella Reference Centre
kD	kilodalton
L1	first-stage-larva
lpg	larvae per gram
MAF	Ministry of Agriculture and Forestry
M., *Mm.*	*musculus*, *musculi* (muscle, muscles)
NBL	newborn larvae
NMRI	Naval Medical Research Institute
OD_{492}	optical density at a wavelength of 492 nm
OIE	Office International des Épizooties, World Organization for Animal Health
OR	odds ratio
p	probability
PCR	polymerase chain reaction
p.i.	post-infection
p.o.	*per os* (by mouth)
RAPD-PCR	random amplified polymorphic DNA polymerase chain reaction
RCI	reproduction capacity index

RLB	reverse line blot hybridization assay
SCVPH	Scientific Committee on Veterinary Measures Relating to Public Health, European Union
SD	standard deviation
SSCP	single-strand conformational polymorphism technique
Taq	polymerase enzyme from *Thermus aquaticus*
T1	genotype synonym to *Trichinella spiralis*
T2	genotype synonym to *Trichinella nativa*
T3	genotype synonym to *Trichinella britovi*
T4	genotype synonym to *Trichinella pseudospiralis*
T5	genotype synonym to *Trichinella murrelli*
T6	genotype 6 of genus *Trichinella*, not species status
T7	genotype synonym to *Trichinella nelsoni*
T8	genotype 8 of genus *Trichinella*, not species status
T9	genotype 9 of genus *Trichinella*, not species status
T10	genotype synonym to *Trichinella papuae*
UN	United Nations
WHO	World Health Organization

3. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, referred to in the text by their Roman numerals.

I Oivanen L, Mikkonen T, Sukura A. An outbreak of trichinellosis in farmed wild boar in Finland. APMIS 2000, 108, 814–818.

II Oivanen L, Kapel CMO, Pozio E, La Rosa G, Mikkonen T, Sukura A. Associations between *Trichinella* species and host species in Finland. Journal of Parasitology 2002, 88, 84–88.

III Kapel CMO, Oivanen L, La Rosa G, Mikkonen T, Pozio E. Evaluation of two PCR-based techniques for molecular epidemiology in Finland, a high-endemic area with four sympatric *Trichinella* species. Parasite 2001, 8, S39–S43.

IV Oksanen A, Oivanen L, Eloranta E, Tirkkonen T, Åsbakk K. Experimental trichinellosis in reindeer. Journal of Parasitology 2000, 86, 763–767.

V Oivanen L, Mikkonen T, Haltia L, Karhula H, Saloniemi H, Sukura A. Persistence of *Trichinella spiralis* in rat carcasses experimentally mixed in different feed. Acta Veterinaria Scandinavica 2002, 43, 203–210.

4. INTRODUCTION

Trichinellosis is a zoonotic disease that can be passed from animals, both wild and domestic ones, to humans. The causative agent is a parasitic nematode of the genus *Trichinella*. These parasites have a wide range of host species, mostly mammals. The infection is contracted by eating raw or inadequately cooked meat of an infected animal. The lifecycle of *Trichinella* is domestic when the infection is passed among domestic animals and rats, and sylvatic when passed among wildlife away from human habitation. Trichinellosis is endemic when the infection is constantly present in a particular area.

Trichinellosis has been described as an emerging and/or re-emerging disease in Europe and the world during the past decades. New sources of human infection and new species of parasites in the genus *Trichinella* have been identified. (Kim, 1991; Pozio, 1995; Dupouy-Camet, 2000; Pozio, 2001) Infected horses have caused several severe human outbreaks in Italy and France resulting in tightened trichinella control in the European Union (EU) (EEC, 1976a). Another considerable source of human infections in Europe is game meat. The global prevalence of the disease is unknown, but estimates indicate that 11 million people may be infected. The most common sources of human infection worldwide are pork, wild boar, and other game meat. However, horse, dog, and many other animal meats have also transmitted the infection. (Dupouy-Camet, 2000) In Finland, human trichinellosis has not been reported since 1977 (Salmi, 1978; MAF, 1999).

In many western and southern European countries, such as Denmark, Germany, the Netherlands, Great Britain, France, and Italy, *Trichinella* infection in domestic pigs is rare, and detected prevalence in wildlife these days is low (Pozio, 2001). In Finland, the increasing trend in the number of *Trichinella*-infected domestic pigs in the 1980s raised concerns. Since then, sporadic infections in pigs have regularly been found at meat inspection. The infection is endemic in wildlife and the prevalence in some regions of Finland is very high, especially in red foxes and raccoon dogs (80% and 35%, respectively). The prevalence in foxes seems to have increased simultaneously with the number of infections in pigs (Hirvelä-Koski *et al*., 1985; Oivanen and Oksanen, 1994). The role and relevance of wild animal hosts and rats as they relate to pigs have been the topics of much discussion.

To understand the epidemiology of *Trichinella*, more information about species existing in Finland was needed. Climatic, geographical, and agricultural circumstances here differ from those in western and southern Europe. Preventing *Trichinella* infections in domestic animals requires information about local epidemiological factors. Consumer protection against trichinellosis should also be based on knowledge of existing parasite species. In this study, endemic domestic *Trichinella* infections in Finland were examined from epidemiological and experimental perspectives.

5. REVIEW OF THE LITERATURE

5.1 History of trichinellosis

Trichinellosis has threatened human health for thousands of years. The earliest reported infection was in an Egyptian man who lived around 1200 BC. The infection was detected from his mummified body (de Boni *et al*., 1977). The Mosaic ban on pork consumption in the Old Testament may have been based on the threat of trichinellosis (3. Ms. 11, 3; Gould, 1970a). The early Jewish Law of Moses was probably written ca. 900-400 BC.

Trichinella spiralis was identified in London in 1835s the parasite being detected in an autopsy of an Italian male corpse (Owen, 1835). Some earlier observations had been made on calcified muscle larvae without knowledge of what they were (Gould, 1970a; Blancou, 2001). At the time, microscopes were rare, which impeded parasitological studies. The first report of experimental infection was published by the German scientist Herbst in 1851. He fed puppies with badger (*Meles meles*) muscles containing *Trichinella*, after which the dogs became infected (Herbst, 1851). The mechanism of infection and the life cycle were finally elucidated by German scientists Leuckart, Virchow, and Zenker (Virchow, 1859; Leuckart, 1860; Zenker, 1860; Campbell, 1983). The pathologist Zenker discovered the biological, pathogenic, and potentially fatal role of *T. spiralis* in humans (Zenker, 1860; Nöckler *et al.*, 2000). The first to detect *Trichinella* larvae in pork was American scientist Leidy (Leidy, 1846; cited by Gould, 1970a). He noted that the parasites were similar to those detected in dead human bodies.

5.2 Taxonomy

The taxonomy of the genus *Trichinella* (Owen, 1835; Railliet, 1896) has been presented with slightly varying details (Soulsby, 1982; Noble *et al*., 1989). According to the traditional classification, the genus belongs to the phylum *Nematoda*, roundworms, class *Adenophorea*, order *Trichinellida*, and superfamily *Trichinelloidea* (Noble *et al*., 1989). The taxonomy has recently been challenged. On the basis of results from ribosomal deoxyribonucleic acid (DNA) sequences, the present higher-level classification of *Nematoda* will need revision into two classes, *Secernentea* and *Adenophorea* (Blaxter *et al*., 1998).

5.2.1 Species and genotypes

For more than 100 years since the discovery of *T. spiralis*, trichinellosis was commonly assumed to always be caused by the unique parasite species. Observations of African isolates revealed that different geographical isolates differed in infectivity to pigs and rats (Nelson and Mukundi, 1963; Nelson *et al*., 1966). In 1972, a new species was described: the nonencapsulated *Trichinella pseudospiralis* (Garkavi, 1972). The same year, Russian scientists suggested that the African strain and those originating from temperate areas could be separate species: *Trichinella nelsoni* and *Trichinella nativa*, respectively, based on differences in infectivity and pathogenicity (Britov and Boev, 1972). However, many scientists

questioned the existence of separate species for years (Madsen, 1976). Differences in infectivity and cross-breeding capability between *Trichinella* isolates from Alaska, Canada, and Kenya were reported (Sukhdeo and Meerovitch, 1977; Kjos-Hanssen, 1984). The freezing resistance of arctic strains was also discovered (Dick and Belosevic, 1978; Dies, 1980; Kjos-Hanssen, 1983).

In the early 1990s, the genus *Trichinella* was divided into eight distinct gene pools. The classification was based on allozyme analysis of 152 isolates at the International *Trichinella* Reference Center (ITRC). The gene pools were assigned with codes T1-T8 (La Rosa *et al*., 1992). The taxonomy of *Trichinella* was soon revised further by comparing reported data on approximately 300 different strains. The genus was divided into five separate species and into three additional taxonomic groups or genotypes (Pozio *et al*., 1992a; Pozio *et al*., 1992b). During the past few years three new species and one more genotype have been described (Nagano *et al*., 1999; Pozio *et al*., 1999a; Pozio and La Rosa, 2000; Pozio *et al*., 2002a). At the moment, the genus includes eight taxa with species status and three genotypes without species status (Murrell *et al*., 2000; Pozio *et al*., 2002a). Species identification is not based on morphological characters, of which there are only few useful, but rather on biological, molecular, and biochemical markers. The main biological markers are host range, temperature tolerance, and number of newborn larvae (NBL) produced by females in specific hosts, while the most striking morphological marker is the collagen capsule that surrounds the muscle-stage larva-nurse cell complex; five of eight species possess this marker. These encapsulated species are *T. spiralis*, *T. nativa*, *T. britovi*, *T. nelsoni*, and *Trichinella murrelli*. The nonencapsulated species are *T. pseudospiralis*, *Trichinella papuae*, and *Trichinella zimbabwensis* (Pozio and Bruschi, 2001; Pozio *et al*., 2002a). Based on morphological, biological, zoogeographical, and molecular markers, the genus *Trichinella* has been postulated to include two evolutionary lines and should therefore be divided into two separate genera: encapsulated and nonencapsulated (Pozio *et al*., 2001a; Rombout *et al*., 2001; La Rosa *et al*., 2003).

At present, the primary methods for identifying *Trichinella* species are based on molecular techniques, although isoenzyme analysis is sometimes used as well (Zarlenga and La Rosa, 2000).

5.2.1.1 *Trichinella spiralis*

This species was first detected by medical student James Paget and described as *Trichina spiralis* by Richard Owen in England in 1835 (Owen, 1835; Paget, 1866). Railliet changed the name to *Trichinella spiralis* since the name *Trichina* had already been given to a genus of insects in 1830 (Railliet, 1896). *Trichinella spiralis* has high infectivity for humans, domestic pigs (*Sus scrofa*), rats (*Rattus* spp.), and mice (*Mus musculus*) (Nelson and Mukundi, 1963; Kozar and Kozar, 1965, Dick and Belosevic, 1978; Kjos-Hanssen 1984; Pozio *et al*., 1992a), but it is also infective for horses (*Equus caballus*) (Arriaga *et al*., 1995) and several wild mammal hosts (Dame *et al*., 1987; Murrell *et al*., 1987; La Rosa *et al*., 1992). The species occurs worldwide and is also known as genotype T1 (La Rosa *et al*., 1992; Pozio, 2000).

Typical characters are high NBL production per female worm *in vitro*, high reproduction capacity index (RCI) in Wistar rats, and early encapsulation in mouse muscles (Pozio *et al*., 1992a). It has been reported to have no resistance to freezing (Pozio *et al*., 1992a), but conflicting observations have also been published (Theodoropoulos *et al*., 2000; Malakauskas and Kapel, 2003).

5.2.1.2 *Trichinella nativa*

Trichinella nativa, named by Britov and Boev (Britov and Boev, 1972), is also known as genotype T2 (La Rosa *et al*., 1992). This species is very widespread in arctic and subarctic areas of the northern hemisphere (Pozio, 2000). A typical character of the species, demonstrated by many researchers (Dick and Belosevic, 1978; Dies, 1980; Chadee and Dick, 1982; Kjos-Hanssen, 1983; Malakauskas and Kapel, 2003), is its high resistance to freezing, especially in carnivore host tissue. The species has been found in many different wild carnivorous and omnivorous mammal hosts, including bears, polar bears, foxes (La Rosa *et al*., 1992), and wild boars (Pozio and Kapel, 1999), as well as in pigs, dogs, and humans (La Rosa *et al*., 1992; Gasser *et al*., 1998; Schellenberg *et al*., 2003). Contrary to T1, this species is only slightly infective for rats and swine (Pozio *et al*., 1992a; Kapel *et al*., 1998; Malakauskas *et al*., 2001).

5.2.1.3 *Trichinella britovi*

Trichinella britovi, genotype T3, was described over a decade ago (La Rosa *et al*., 1992, Pozio *et al*., 1992b). It occurs in Eurasia, in many areas with *T. spiralis* (Pozio, 2000). The species differs from *T. spiralis* with weak infectivity for rats, moderate resistance to freezing, moderate infectivity for swine, slow nurse cell development and low *in vitro* production of NBL (Pozio *et al.*, 1992a; Pozio *et al.*, 1992b; Malakauskas and Kapel, 2003). The species has been found in many wild carnivores, such as red foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutes procyonoides*), but it can also infect horses, wild boars, domestic pigs, and humans (Pozio *et al*., 1992b; Kapel *et al*., 1998).

5.2.1.4 *Trichinella nelsoni*

Trichinella nelsoni, genotype T7, was detected in Kenya (Nelson *et al*., 1963) and described first by Britov and Boev (Britov and Boev, 1972; La Rosa *et al*., 1992). The species occurs in equatorial and southern Africa (Pozio, 2000), mainly in wild carnivorous hosts such as hyenas (*Hyaenidae*) and felines (*Felidae)* (Murrell *et al.*, 2000). It has occasionally been detected in pigs (*Suidae*) and humans, although it has very low infectivity for pigs and rats (Nelson *et al*., 1963). The infectivity for humans has not been confirmed (Pozio, 2001). The species has low NBL production *in vitro* and extended nurse cell production in mice (Pozio *et al*., 1992a; Pozio *et al*., 1992b). It has no resistance to freezing (Pozio *et al*., 1992a) but is unusually tolerant of high temperatures. The muscle larvae can withstand a temperature of 56°C for 60 minutes (Boev and Sokolova, 1981).

5.2.1.5 *Trichinella murrelli*

A relatively new species addition to the genus is *T. murrelli*, genotype T5 (La Rosa *et al*., 1992). This was known as a North American genotype until its recognition as a separate species (Pozio and La Rosa, 2000). It occurs in North America (Pozio, 2000). *Trichinella murrelli* mainly has wild carnivorous mammalian hosts, but it has also been found in horses and humans. This species has very low reproductive capacity in pigs and rats, low NBL production *in vitro*, slow nurse cell development, and low resistance to freezing (Pozio and La Rosa, 2000; Malakauskas and Kapel, 2003).

5.2.1.6 *Trichinella pseudospiralis*

Trichinella pseudospiralis has been recognized as a species since 1972 (Garkavi, 1972). The species is also known as genotype T4 (La Rosa *et al*., 1992). *Trichinella pseudospiralis* can infect many mammals, including humans, but also birds, unlike other *Trichinella* species (Shaikenov, 1980; Shaikenov and Boev, 1983; Ainsworth *et al*., 1994). The species occurs worldwide (Pozio, 2000). The length of larvae and adults is smaller than in other species, and the reproductive capacity is high in rats but low in pigs (Pozio *et al*., 1992b; Pozio *et al*., 1992c). The species is generally believed not to resist freezing (Pozio *et al*., 1992a), but according to some reports, certain strains can survive for short periods (Theodoropoulos *et al*., 2000; Malakauskas and Kapel, 2003). On the basis of its wide host range, extensive distribution, and the absence of the capsule it has been hypothesized to be the most ancient species in the genus *Trichinella* (Pozio *et al*., 1992c). In a comparison of *T. pseudospiralis* strains from different parts of the world, three genotypic isolates were identified by multiplex polymerase chain reaction (PCR) test (Zarlenga *et al*., 1999; La Rosa *et al*., 2001).

5.2.1.7 *Trichinella papuae*

Trichinella papuae, T10, has been found only in Papua New Guinea in southeast Asia, where it has been detected in swine and wild boars. It can also infect humans, laboratory mice, red foxes, turtles (*Pelomedusa subrufa*), pythons (*Python molorus bivittatus*), varans (*Varanus exanthematicus*), and caimans (*Caiman* sp.) (Jongwutiwes *et al*., 1998; Pozio *et al*., 1999a; Pozio, 2001; Webster *et al*., 2002, Pozio *et al*., 2004a), but it does not infect birds (La Rosa *et al*., 2001). The length of its muscle larvae is greater than that of *T. pseudospiralis* (970 µm in males, 1,000 µm in females) (Pozio *et al*., 1999a). Muscle larvae are non-encapsulated and lack freezing tolerance but can survive in +5°C storage for four weeks (Webster *et al*., 2002).

5.2.1.8 *Trichinella zimbabwensis*

The latest species in the genus, *T. zimbabwensis*, has been detected in farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe. It was described as a species in 2002 (Foggin *et al*., 1997; Mukaratirwa and Foggin, 1999; Pozio *et al*., 2002a). It is the first *Trichinella* strain isolated in reptiles in nature. In the laboratory, it can also infect rats, mice, pigs, baboons (*Papio* sp.), turtles, pythons, varans, and caimans. Its muscle larvae are non-encapsulated. It is not infective for birds, nor can it resist freezing (Pozio *et al*., 2002a; Pozio *et al*., 2004a).

5.2.1.9 Genotypes of uncertain taxonomic status

Genotype T6 is quite similar to *T. nativa*. The muscle larvae of both genotypes resist freezing well. Genotype T6 has been detected only in wild hosts of certain areas of North America (Worley *et al*., 1986; La Rosa *et al*., 1992; Pozio *et al*., 1992a; Murrell *et al*., 2000). Genotype T8 has been identified in a few isolates from wild carnivores of southern Africa. The genotype can be distinguished by some molecular markers for *T. nelsoni* and *T. britovi*, which seem to be close to this genotype (La Rosa and Pozio, 2000). *Trichinella* genotype T9 has been identified in Japan (Nagano *et al*., 1999) and appears to be related to *T. britovi*. According to fairly recent evidences, T8 and T9 may be subspecies of *T. britovi*, and T6 a subspecies of *T. nativa* (Zarlenga and La Rosa, 2000).

5.3 Life cycle and morphology

5.3.1 Life cycle

The basic life cycle of *Trichinella* has been known since the middle of the 19th century. This genus is unique among parasitic nematodes in that all stages of the life cycle occur within a single host. In nature, the cycle is repeated when another host animal ingests the flesh of another host containing viable muscle-stage larvae (Villella, 1970) (Figure 1). The life cycle of trichinellae follows the five-stage four-molt pattern of other nematodes. The NBL penetrate the intestine, invade the muscles, and encapsulate without molting. All four molts take place in the intestine of the new host. The life cycle consists of the intestinal phase with molting and the parenteral phase with muscle-infecting first-stage larva (L1) (Kozek, 1971a; 1971b).

Ingested muscle larvae are released from the surrounding tissue and their capsules by the action of pepsin and hydrochloric acid in the stomach of the new host. The larvae pass into the small intestine, invade the epithelial wall, and penetrate a row of columnar epithelial cells. The larvae become sexually mature in about 30 hours. After copulation, the females begin to produce NBL on the sixth or seventh day post-infection (p.i.). The process continues until the immune response forces their expulsion from the intestine, usually until the end of the sixth week p.i . (Villella, 1970; Kozek, 1971a; Despommier, 1983; Capó and Despommier, 1996; Bruschi and Murrell, 2002). The intracellular stage, molting, ecdysis, and reproduction of *T. spiralis* have been studied *in vitro* by intestinal epithelial cell cultures. More than 50% of the parasites develop to adult stages and survive for nine days (Gagliardo *et al*., 2002). The number of NBL shed depends on the immune status of the infected host, host species, and on the infecting species of *Trichinella* (Pozio *et al*., 1992a; Pozio *et al*., 1992b; Capó and Despommier, 1996). One female adult worm may produce as many as 500-1500 new larvae. The time period from the larvae being released from the cyst until production of the new generation is called the enteral or intestinal phase of the life cycle. The mean sexual ratio between *T. spiralis* males and females has been reported to be 1.82:1 (range 1.72-2.59:1) (Liu *et al*., 1991).

At the beginning of the parenteral or muscular phase most of the NBL penetrate into the submucosa and invade through the circulation or lymph vessels to various organs and

skeletal muscles. The larvae entering the striated muscles survive and penetrate the muscle cells. With encapsulating species, the larvae become coiled and the host cells are modified into nurse cells. The term nurse cell is used because this modulated host cell aids the parasite in obtaining nutrients and exporting wastes. The larvae of encapsulating *Trichinella* species induce formation of a collagen capsule around nurse cells (Despommier, 1990; Capó and Despommier, 1996; Pozio and Bruschi 2001; Bruschi and Murrell, 2002). Using interleukin-10 knockout mice, it has been recently demonstrated that interleukin-10 protects the liver from damage and inflammation caused by migrating NBL (Bliss *et al*., 2003). Interleukin-10 has also been shown to limit the inflammatory response to muscle infection caused by *Trichinella* larvae. Beiting and others (2004) concluded that the immune response is controlled in order to promote the survival of both parasite and host. The finding supports the idea of developed adaptation between the host and parasite (Beiting *et al*., 2004). The time needed for capsule formation depends on the parasite and host species involved, being a minimum of 16 days p.i. with *T. spiralis.* Capsule formation promotes the ability of muscle larvae to pass the infection on to the next host. (Pozio *et al*., 1992a; Capó and Despommier, 1996).

The larvae remain in the nurse cells but are not dormant (Despommier, 1990). Generally, they are alive in the capsules for many years. The nurse cell-larva unit may survive in some hosts for the life of the animal. Living *T. spiralis* larvae have been found 39 years after infection in the muscles of a human patient (Fröscher *et al*., 1988).

After the death of the host, the parasite remains infective for weeks or even longer (Despommier, 1975; 1990; Despommier *et al*.,1991). Sometimes both the parasite and the nurse cell die and become calcified. With *T. spiralis*, calcification begins at 2-20 months p.i., the time depending on the host species (Gould, 1970b).

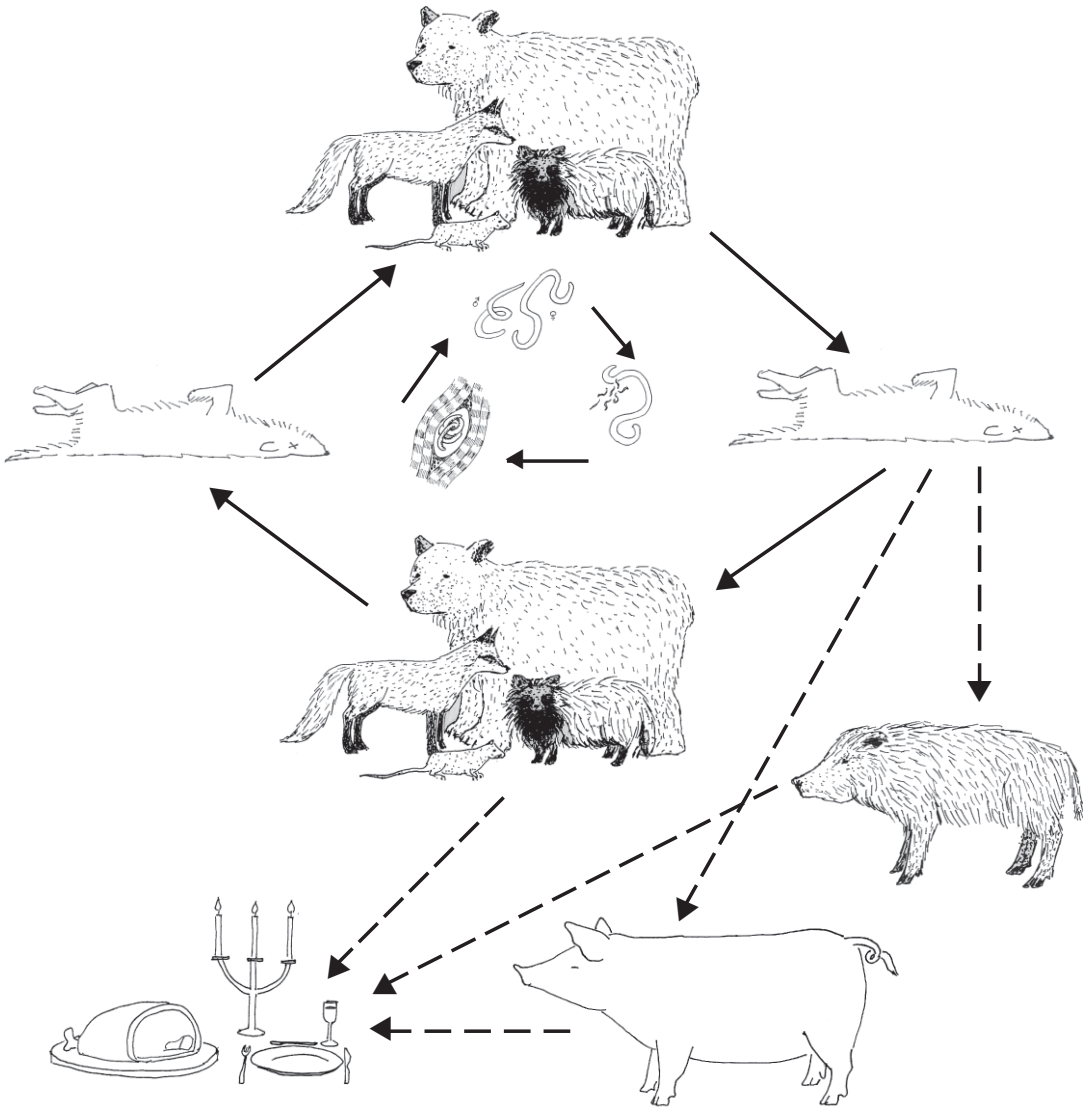

Figure 1. Schematic drawing of the life cycle of *Trichinella* sp. in Finland.

5.3.2 Morphology of the parasite

The length of *T. spiralis* NBL is 80-120 µm and the diameter 5-6 µm. The larvae do not increase in size until they penetrate the muscle cells. The larvae begin to grow in their nurse cells, reaching a length of 900-1280 µm and a diameter of 35-40 µm by 30days p.i. *Trichinella* adult females are a little longer and thicker than the males. Their length and diameter are 2460-3390 µm and 35-70 µm, respectively, while the corresponding figures for males are 1040-1300 µm and 29-32 µm (Villella, 1970; Gould, 1970b). The other capsulating species of the genus do not differ morphologically from *T. spiralis* and have similar dimensions (Lichtenfels *et al*., 1983).

The collagen capsules surrounding the muscle-stage larva-nurse cell complex in the encapsulating species are oval, with a variable size. The variation was earlier believed to depend only on the host species (Gould, 1970b; Madsen 1961), but current data suggest that it also depends on the *Trichinella* species involved (Sukura *et al*., 2002). Usually only one larva is contained within the capsule, but sometimes several are present (Owen, 1835; Gould, 1970b).

The muscle cell undergoes changes and transforms into a nurse cell after the L1 larva has infected it. These changes include loss of contractile elements, nuclear enlargement with a nucleolus, hypertrophy of the sarcoplasmic reticulum, and vacuolation of mitochondria. The new nurse cell continues changing, with hyperinvolution of the plasma membrane, development of a host-derived double-unit membrane directly adjacent to the entire surface of the larval cuticle, and elaboration of rough endoplasmic reticulum and polyribosomes in the regions near the larval cuticle and the nurse cell plasma membrane (Despommier, 1975).

The changes in the infected muscle cell of nonencapsulating species resemble those in encapsulating species. The nonencapsulating *T. papuae* also induces changes in the muscle cell. In addition, it induces clustering of mitochondria around the muscle larva (Pozio *et al*., 1999a). Two other nonencapsulating species, *T. zimbabwensis* and *T. pseudospiralis*, induce changes in the muscle cell as well. The cytoplasm seems structureless and the mitochondria vacuolated. No infiltration of inflammatory cells has been seen around the muscle larvae (Pozio *et al*., 2002a; Pozio *et al*., 2004a).

5.4 Pathogenesis and clinical signs

The clinical signs of acute trichinellosis are characterized by two phases: an enteral and a parenteral phase, corresponding to the presence of parasites in the intestine and in the circulation and/or musculature, respectively. The severity of the clinical course depends firstly on parasitic factors, such as the species involved and the number of infective larvae ingested, and secondly on host factors, such as sex, age, and immune status (Pozio *et al*., 1993; Capó and Despommier, 1996; Bruschi and Murrell, 2002).

5.4.1 Clinical signs

Typical of trichinellosis outbreaks is that most people who become infected are asymptomatic. The most common signs during the enteral phase of a mild infection are transient diarrhea and nausea. However, in moderate to severe infections, the first signs are upper abdominal pain, diarrhea or constipation, vomiting, malaise, and mild fever. The enteral phase lasts for six weeks p.i. (Capó and Despommier, 1996; Kocięcka, 2000; Bruschi and Murrell, 2002).

From the second to the sixth week p.i., the enteral phase is still present, but the dominating signs arise from the parenteral phase due to the migrating larvae and their indiscriminate penetration of different tissues. During the third week p.i. the symptoms intensify due to invasion of muscle cells. Characteristic signs include weakness, pain, paralysis, and photo-

phobia. Edema is prominent and patients may have shortness of breath. Endocarditis, myocarditis, and cardiac failure have been reported. The signs of acute illness usually diminish from the fifth or sixth week p.i. onwards (Gould, 1970c; Capó and Despommier, 1996).

In mild or moderate infections, the most typical symptoms are diffuse myalgia, fever, periorbital or facial edema, headache, conjunctivitis, and skin rash. Other possible signs are paralysis-like state, difficulties in swallowing, insomnia, weight loss, peripheral nerve sensations, hot flashes, coryza, bronchitis, hoarseness, splinter hemorrhages of the nail beds and/or retinae, visual disturbances, and paralysis of the ocular muscles (Capó and Despommier, 1996; Kocięcka, 2000; Bruschi and Murrell, 2002).

In severe infections, the symptoms are prominent. These patients are first to be diagnosed in an epidemic. Typical signs include high fever, severe muscle pain, skin rash, headache, and edema of the face, eyelids, or extremities. Also neurological signs may occur, such as headache, vertigo, tinnitus, deafness, aphasia, convulsions, and abnormalities related to peripheral reflexes. Meningitis, encephalitis, and hemiplegia may also develop. Underlying the neurological symptoms is damage of the brain tissue due to occlusion of arteries or granulomatous inflammation. The edema is probably the result of an allergic reaction (Capó and Despommier, 1996; Kocięcka, 2000; Bruschi and Murrell, 2002). Even delirium and psychosis have been reported (Dalessio and Wolff, 1961).

Possible complications include abortion and intrauterine infection of the fetus (Gould 1970c; Dubinský *et al*., 2001). In extensive infections, the patient may die, typically between the third and the fifth week p.i., due to heart failure, central nervous system failure, myocarditis, encephalitis, pneumonitis, hypokalemia, adrenal gland insufficiency, or obstruction of blood circulation (Gould, 1970c; Capó and Despommier, 1996). Some patients have long-term symptoms, such as generalized myalgia, ocular symptoms, and neuropathies, years after the infection (Harms *et al*., 1993; Capó and Despommier, 1996).

5.4.2 Laboratory findings

The most characteristic hematological finding is peripheral eosinophilia during the enteral phase. This reaches the maximum level on the third and fourth weeks. Even among asymptomatic cases, the eosinophil count is elevated. Other common findings are elevated levels of the muscle enzymes creatinine phosphokinase, 1,6-diphosphofructoaldolase, lactate dehydrogenase aldolases, and aminotransferases (Capó and Despommier, 1996).

5.4.3 Acquired immunity

According to research among the Canadian Inuit people, secondary infection differs from primary infection. The IgG response is rapid and the IgM response weaker. Primary infection is characterized by delayed IgG response and an intense IgM response. Patients with a secondary infection often have diarrheic syndrome without edema and less frequently, fever or rash (MacLean *et al*., 1992).

5.4.4 Infective dose

Exact data on the minimum or average infective dose for humans are unavailable. Estimates of 50-500 larvae have been presented (Casarosa, 1985, cited by Battelli *et al*., 1994; Murrell, 1985). Infective doses for patients of three epidemics in Italy were retrospectively estimated to be 300-30 000 larvae of *T. britovi* or 5000-18 000 larvae of *T. spiralis* (Pozio *et al*., 1993). According to the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) of the European Union (EU), the minimum infective dose for *T. spiralis* is 100-300 ingested larvae (SCVPH, 2001). The fatal infection dose for humans is estimated to be 5 larvae per gram (lpg) body weight, and for swines 10 lpg body weight (Chandler and Read, 1961, cited by Olsen *et al*., 1964).

5.4.5 *Trichinella* species-specific pathogenicity in humans

The severity of the clinical course and the presenting signs depend on the species of *Trichinella* involved. In humans, *T. britovi* infection appears to cause milder symptoms during the intestinal phase than *T. spiralis* (Pozio *et al*., 1993). In France, the frequency of facial edema in *T. murrelli* infections was reported to be lower than that observed in *T. spiralis* infections (Dupouy-Camet, 1993, PhD thesis, cited by Bruschi and Murrell, 2002). Infections caused by *T. pseudospiralis* are related to prolonged muscle symptoms; these symptoms can continue for years (Ainsworth *et al*., 1994; Jongwutiwes *et al*., 1998). *Trichinella spiralis, T. nativa, T. pseudospiralis,* and *T. murrelli* have been reported to cause mortality in humans (Jongwutiwes *et al*., 1998; Dupouy-Camet, 1993, PhD thesis, cited by Bruschi and Murrell, 2002). *Trichinella spiralis* is considered to be the most pathogenic for humans, *T. nativa* shows moderate pathogenicity and *T. nelsoni* low pathogenicity (Capó and Despommier, 1996).

5.4.6 Host-specific pathogenicity

Host species and responsiveness influence the infectivity and pathogenicity of the parasite (Wakelin and Goyal, 1996). Clinical signs of trichinellosis and infection severity differ according to the host species involved. Contrary to man, some mammals do not develop noticeable clinical signs during *Trichinella* infection. This has, for instance, been observed in swine with infection doses of 2.2-6.6 lpg body weight (Olsen *et al*., 1964). In experiments with raccoon dogs, virtually no clinical symptoms appeared after *Trichinella* infection with one larva per gram body weight (Näreaho *et al*., 2000). However, a case report of a dog showed severe neuromuscular symptoms apparently due to *Trichinella* infection (Lindberg *et al*., 1991).

Different *Trichinella* species are adapted to different hosts. In some host species, such as humans, mice and guinea pigs, several *Trichinella* species can breed succesfully. They are more versatile hosts, such as humans, mice, and guinea pigs. Other hosts, such as swine and rats, are more selective (Pozio *et al*., 1992b; Capó and Despommier, 1996; Webster *et al*., 1999; Kapel, 2000; Malakauskas *et al*., 2001).

Muscle predilection of the parasite varies according to host species (Hermansson, 1943; Fassbender and Meyer, 1974; Kapel *et al*., 1994; Kapel *et al*., 1998; Serrano *et al*., 1999; Gamble *et al*., 2000; Nöckler *et al*., 2000). The relative distribution of the parasites in the musculature may also depend on infection level (Christensson and Lunsjö, 1994; Serrano *et al*., 1999). In humans, the most frequently infected muscles include the diaphragm, *Musculus gluteus maximus*, *Musculi pectoralii*, and *Musculus deltoideus* (Gould, 1970b). In swine, *T. spiralis* prefers the tongue, diaphragm, masticatory muscles, and intercostals (Olsen *et al*., 1964; Gamble, 1996; Forbes and Gajadhar, 1999; Serrano *et al*., 1999), and *T. britovi* the tongue, diaphragm, neck, and masticatory muscles (Kapel *et al*., 1998). The predilection sites in wild boar (*Sus scrofa*) are the tongue and diaphragm. Some authors have noticed slight differences in predilection muscles according to parasite species, while others have not (Serrano *et al*., 1999; Kapel, 2001). The predilection muscles are roughly the same in horses, sheep, and brown rats (Machnicka-Roguska, 1969; Alkarmi *et al*., 1990; Tomašovičová *et al*., 1991; Gamble *et al*. 1996, Pozio *et al*., 1999b; Theodoropoulos *et al*., 2000). The preferred muscles in carnivores are slightly different from those in herbivores (Kapel *et al*., 1995; Mikkonen *et al*., 2001).

5.5 Therapy

In practice, pharmaceutical treatment is used only with human patients, not with animals. In mild infections, there is no need for medical treatment. At the early stage of more serious infections, the pharmacotherapy can limit the muscle invasion of larvae. During the later stages, the aim is to reduce muscle damage and general symptoms (Bruschi and Murrell, 2002).

5.5.1 Anthelmintic drugs

The most often chosen anthelmintic is the benzimidazole derivative mebendazole, but albendazole and thiabendazole are also used. Medication taken at the early stage of the infection gives the best result. Mebendazole is poorly absorbed from the intestinal lumen, but it is well tolerated. Albendazole is absorbed from the alimentary tract and is also well tolerated. While thiabendazole is considered an effective drug, it has numerous side-effects. All of these drugs are contraindicated in pregnant women and young children (Kocięcka, 2000; Bruschi and Murrell, 2002). Treatment with mebendazole showed no effect on *T. spiralis* larvae encapsulated in the muscles. Mebendazole is effective and recommended for treating NBL (Pozio *et al*., 2001b). In an outbreak caused by *T. pseudospiralis*, patients continued to suffer from muscle symptoms at four months p.i., despite treatment with mebendazole and thiabendazole. However, they recovered well after changing the drug to albendazole (Jongwutiwes *et al*., 1998). Pyrantel has been used to treat pregnant women and young children. It is not absorbed from the intestinal lumen and acts by paralyzing parasites (Kocięcka, 2000).

5.5.2 Immunosuppressive drugs and adjunct therapy

Glycocorticosteroids are used in the acute stage of trichinellosis to suppress signs of immediate-type hypersensitivity. However, their application in therapy has not been tested in a convincing clinical trial. They should not be used without anthelmintic therapy, as they could increase the larval burden by delaying the usual expulsion of intestinal worms (SCVPH, 2001). Other treatment of seriously ill persons includes hospitalization, rehydration, and administration of analgesic drugs (Kocięcka, 2000).

5.5.3 Prophylaxis

Experiments have been conducted on vaccination against trichinellosis. Pigs and rodents have been immunized with, for instance, living *T. nativa* larvae, killed *T. spiralis* muscle larvae and NBL, or soluble antigens of homogenized adult *T. spiralis*. While offering some protection, it has been insufficient (Smith, 1987; Madden and Murrell, 1990; Xu *et al*., 1994; Zhu *et al*., 1994). Some recent studies were done on immunization with tyvelose, a larval glycoprotein antigen of *T. spiralis*. It failed to confer protection against infection (Goyal *et al*., 2002). At present, the immunoprophylaxis of *Trichinella* infection in pigs is under investigation, but thus far there is no widespread use of vaccines (Bruschi, 2002; Gamble, 2001).

5.6 Diagnostic methods

Diagnostic methods are applied in human medicine, in veterinary public health services, and in scientific surveys. These methods are also needed to satisfy trade requirements of meat. Trichinellosis can be diagnosed using direct or indirect techniques. With direct methods, first-stage muscle larvae are visualized by microscopic examination of tissue or digested muscle sample. Indirect methods are based on testing for specific antibodies or other indicators. Both direct and indirect methods are mainly used to detect the *Trichinella* sp. infection at the genus level. Further diagnostics is then needed to identify the organism at species or genotype level.

5.6.1 Diagnostic methods in human medicine

5.6.1.1 Direct detection

Muscle biopsy is a traditional method applied to diagnose trichinellosis. Samples are usually taken from the *M. deltoideus*. Other possible sites are the *Musculus biceps brachii*, *Musculus gastrocnemius*, *M. pectoralis*, *M. gluteus maximus*, and *Musculi intercostali*. Muscle biopsy is recommended only in cases where serological results are unclear. In autopsy, the sampling site is the diaphragm (Gould, 1970b; Bruschi and Murrell, 2002). The biopsy sample can be examined histologically or it may be digested with pepsin and hydrochloric acid. Histological examination may reveal basophilic changes, inflammation of the tissue, and characteristics through which the age of the infection can be determined (Capó and Despommier, 1996; Bruschi and Murrell, 2002). The biopsy sample can be examined by a trichinoscopy method in which the tissue sample is compressed between two glass plates (compresso-

rium) and a projection microscope known as a trichinoscope or a microscope is used. Typically, the biopsy obtained from a living patient is small, about 2-4 mm^3. A negative result does not exclude an infection. Because of small sample sizes, this method has very limited sensitivity (Capó and Despommier, 1996). With a sample size of 4 mm^3, for instance, only very heavy infections of 250 lpg or more can be detected.

5.6.1.2 Indirect detection

Antibody detection tests are useful in diagnosis on about day 12 p.i. and beyond. Immunofluorescence-based assays and enzyme-linked immunosorbent assay (ELISA) are commonly used for IgG antibodies (Capó and Despommier, 1996). IgE class antibodies appear first after infection and are typical of the acute stage of the disease. However, their determination has not been practical. Antibodies of classes IgM, IgG, and IgA appear at the beginning of the second week p.i., and IgG antibodies may persist for several years (SCVPH, 2001).

An indirect hemagglutination test can be used in human diagnostics. Precipitin and bentonite flocculation tests are not as sensitive as the indirect hemagglutination test (Capó and Despommier, 1996). Intradermal immunological tests have also been used in the past, but they can be very insensitive, failing to detect even clinically severe infections (Mäkelä, 1970).

When ELISA and indirect immunofluorescence (IIF) test were compared in human diagnostics, ELISA was more sensitive (van Knapen *et al*., 1982). To analyze the cross-reactions of IIF in patients with different parasitic diseases, the Western blot (immunoblot) technique was used with good results (Robert *et al*., 1996). The most reliable results are obtained when ELISA and IIF tests were combined (SCVPH, 2001). According to recent recommendations, ELISA can be used as the primary diagnostic method. Among the serological methods tested, it proved to be the most sensitive (Gamble *et al*., 2004). The reliability of serological methods is highly dependent on the quality and specificity of the antigens used (Homan *et al*., 1992).

Tests commercially available are the IIF, ELISA, competitive inhibition assay, immunoblotting, counterimmunoelectrophoresis, and latex agglutination (SCVPH, 2001; Yera *et al*., 2003).

5.6.2 Diagnostic methods in veterinary medicine

5.6.2.1 Direct detection

Microscopic diagnostics of trichinellosis has been applied at meat inspection of potential host animals and in epidemiological surveys. The most traditional diagnostic method is trichinoscopy, also known as the “compression” method. It has been used to examine pork since 1863 (Gould, 1970a). The samples of striated muscles are cut into grain-sized pieces, compressed, and examined with a magnifying instrument. The advantages of this method are the low technical requirements for the laboratory; it is a simple procedure that is carried out

with basic equipment. Drawbacks are that the examination is time-consuming and the technician needs training for the microscopic work.

The ability to detect *Trichinella* larvae by direct detection methods depends on the sample site, sample size, host species, parasite species, infection age, sample quality (e.g. frozen or fresh), competence of the analyst, and on the method employed. The sensitivity of the trichinoscopy method strongly depends on the total sample size, which is 0.5 g per pig according to EU directives (EEC, 1976a; 1976b) and 0.2-0.3 g per pig according to the legislation of the Ministry of Agriculture and Forestry in Finland (MAF, 2002). National differences in regulations have existed between EU countries; 14 grain-sized pieces per pig have been cut in Finland, which is in accord with EU directives. In Germany, as many as 56 pieces per pig have to be examined (EEC, 1976a; 1976b; Nöckler *et al*., 2000; MAF, 2002).

Other notable disadvantages of the method are its low sensitivity and weakness in detecting nonencapsulated muscle larvae such as *T. pseudospiralis*. A misdiagnosed case in wild boar meat was reported in Finland (Sukura *et al*., 2001). In Sweden, in a proficiency test of the trichinoscopy method in meat inspection laboratories, 22 of 26 participants failed to detect *T. pseudospiralis* in muscle samples containing on average 4 lpg (Christensson and Pozio, 2004). The weakness in detecting nonencapsulated species of *Trichinella* can also be seen when very fresh infections with nonencapsulated muscle larvae of capsulating species are examined. To detect muscle larvae in the compressorium, the stereomicroscope is a more sensitive device than the trichinoscope (Forbes *et al*., 2003a).

The trichinoscopy method is no longer recommended for meat inspection in EU countries since *T. pseudospiralis* has been reported in several countries including France, Italy, and Finland (SCVPH, 2001). The method was taken out of use in Finland in 2004 (MAF, 2002; 2004c). However, in the past, this method has been used with some success used together with meat processing requirements and education of consumers to protect humans against pork-transmitted trichinellosis (Gajadhar and Gamble, 2000).

The artificial digestion method was applied to *Trichinella* detection as early as in 1897, when larvae were isolated from muscle tissue by pepsin-hydrochloric acid digestion (Thornbury, 1897, cited by Gould, 1970b). The main steps in all modifications of this method in *Trichinella* examination remain the same. In brief, samples of tissue are digested in an artificial gastric fluid containing about 1% pepsin and 1% hydrochloric acid (final concentration 0.12 N). The ground or diced samples are stirred or shaken in the fluid at 40-46°C for 30min or longer. After letting the digest settle, the larvae are detected in the sediment by stereomicroscope or trichinoscope. Usually, a pooled sample of swine muscles, 1.0 g per animal and 100 g in total, is examined simultaneously (EEC, 1976a; 1976b; Gamble, 2001; MAF, 2002).

Several modifications of the pooled sample artificial digestion method have been published. Of these, six have been approved by the EU (EEC, 1976a; 1976b), and of these six, two have been approved in Finland: 1) the Stomacher digestion method using a Stomacher® Lab Blender 3500T and 2) the magnetic stirrer method for pooled samples, which uses a magnetic

stirrer to blend and warm the digestion solution (MAF, 2002). Efforts to develop a validated method and quality system in artificial digestion have led to a novel modification named double separatory funnel digestion. Forbes and Gajadhar (1999) determined 14 critical control points to control adequate test performance step by step.

The artificial digestion method is sensitive to the quality of the samples. Freezing of muscle samples is known to markedly diminish the number of larvae detected (Jackson, 1977; Hirvelä-Koski *et al*., 1985). The effect is probably strongest on the *Trichinella* species that are nonresistant to freezing.

A theoretical sensitivity for testing a 1-g sample with artificial digestion is an infection level of 1 lpg, but the actual sensitivity is closer to 3-5 lpg (Gamble, 1996, 1998, 1999). According to other estimates, the detection limit when examining a 1-g sample is 3 lpg for trichinoscopy and 1 lpg for digestion methods (Nöckler *et al*., 2000; SCVPH, 2001). Comparison of the artificial double funnel digestion method with the trichinoscopy method revealed that the former was 3.2 times more likely to detect infected tissues than the latter. Both methods were tested with 1-g samples (Forbes *et al*., 2003a). With pigs, the standard sample size is 1 g, but with horses at least 5-g samples are recommended (Gamble *et al*., 1996; Boireau *et al*., 2000; Nöckler *et al*., 2000).

The methods applied at routine meat inspection are primarily aimed at preventing clinical human trichinellosis. For this purpose, it is necessary to ensure a minimum sensitivity of 1-3 lpg for tissue taken from the predilection site. Infections of >1 lpg in meat are generally considered a public health concern (Gamble, 1996; Nöckler *et al*., 2000; SCVPH, 2001).

5.6.2.2 Indirect detection

The indirect serological diagnostic methods can be used at both antemortem and postmortem examination for *Trichinella*-specific antibodies. Several conventional serodiagnostic methods have been applied in detecting *Trichinella* larvae. These include ELISA, immunofluorescence antibody test (IFAT), Western blot analysis, complement fixation test, and hemagglutination test (Nöckler *et al*., 2000).

The ELISA method has been applied in *Trichinella* diagnostics for several decades (Ruitenberg and Van Knapen, 1977; Van Knapen *et al*., 1980; Ruitenberg *et al*., 1983). As the antigen for ELISA, crude larval extract and excretory-secretory (ES) products of *in vitro* cultivated *Trichinella* larvae have been used. The latter has been shown to be more specific when examining pigs. False-positive ELISA reactions have been linked to pigs infected by other nematodes. Improvement of antigen quality has increased specificity. The World Organization for Animal Health (OIE) recommends using secreted stichosome antigens collected from *Trichinella* larvae. These consist of a group of structurally related glycoproteins with molecular weights of 45-53 kD (Gamble *et al*., 1983; Gamble and Graham, 1984; Gamble *et al*., 1988; Gamble, 2001). A synthetic glycan antigen has also been developed for use in ELISA. It has been reported to increase the sensitivity and specificity of the test (Gamble *et al*., 1997). In

addition to muscle-stage larvae, diagnostic components can be found in *Trichinella* adults and NBL (Appleton *et al*., 1991).

Traditionally ELISA has been applied to analyze antibodies in serum samples. According to some reports, samples of muscle juice can substitute for serum samples. This may be a practical solution if serum is unavailable. Results with muscle juice were promising in pigs but inconsistent in wild red foxes (Kapel *et al*., 1998; Vercammen *et al*., 2002).

The ELISA method is relatively simple to apply, and it can be automated in *Trichinella* diagnostics. It is sufficiently sensitive to detect low-level infections (Nöckler *et al*., 2000). An experiment with *T. nativa*-infected pigs showed high antibody response with ELISA, although no muscle larvae were recovered with digestion method (Kapel *et al*., 1998).

ELISA is recommended for herd surveillance programs and can be used in detecting ongoing transmission of *Trichinella* at farm level (Gamble, 1996). However, this method cannot replace the direct methods at meat inspection because it can fail to detect early or very late stages of infections (Nöckler *et al*., 2000; Gamble *et al*., 2004). Some commercial applications of ELISA have been developed (Patrascu *et al*., 2001).

Western blotting is another serological method often applied in *Trichinella* diagnostics (Kapel *et al*., 1998; Yepez-Mulia *et al*., 1999; Pozio *et al*., 2002b). An evaluation of the success of ELISA and Western blot methods in detecting anti-*Trichinella* IgG in horses did not give encouraging results for meat inspection. The artificial digestion method was recommended to be used instead. The serological methods failed to detect infections older than 22-23 weeks, although the muscle larvae were still infective. In horses, the serology of the infection differs from that in some other animals such as pigs (Voigt *et al*., 1997; Pozio *et al*., 2002b). However, ELISA and Western blot methods could detect mild infections that were below the detection level of direct methods, and thus, could be used as supplemental tests to diagnose horse trichinellosis (Yepez-Mulia *et al*., 1999).

The antigen used influences the specificity of serological tests. Somatic antigens, such as crude larval extracts, may cross-react with antigens of other nematodes. Poor quality of serum or blood samples, e.g. samples with extensive hemolysis or bacterial growth, may decrease both the specificity and the sensitivity of the tests. Individual immune response, presence of maternal antibodies, and immunodeficiency syndromes also influence the results. The time of seroconversion varies according to the infective dose, infecting species, and host species. The persistence of antibodies is different in different hosts. When serological methods are used, attention should be paid to validation and quality assurance (Nöckler *et al*., 2000; Gamble *et al*., 2004).

Commonly applied diagnostic methods have different detection limits. The estimated limits for infection intensity in tissue are 0.1 lpg with immunofluorescence tests, 0.01 lpg with ELISA techniques, and 0.001 lpg with PCR (Gamble *et al*., 1983; Nöckler *et al*., 2000; SCVPH, 2001).

Many of the conventional serological methods are laborious and cannot be used in the automated systems needed in meat inspection. However, because serological methods allow testing of living animals, they may be useful for establishing *Trichinella*-free areas and/or farms in the EU and reducing restrictions in international animal trade (Nöckler *et al*., 2000; SCVPH, 2001; Gamble *et al*., 2004).

5.6.3 Species specification and genotyping

Species and genotype detection in the genus *Trichinella* has been based on many criteria, including ecological, biological, and zoogeographical characteristics. Efforts to use antibody responses to differentiate among genotypes have been made but have met difficulties (Zarlenga and La Rosa, 2000).

Restriction enzyme analysis and DNA probes have been applied to identify genotypes. Restriction fragment length polymorphism (RFLP) has been used to differentiate *Trichinella* genotypes and isolates and to investigate the epidemiology of trichinellosis (Dame *et al*., 1987; Zarlenga and La Rosa, 2000).

The application of allozymic analysis in typing of 152 isolates revealed at least eight distinct gene pools (La Rosa *et al*., 1992). This isoenzyme analysis technique has played an important role in characterizing *Trichinella* isolates in epidemiological surveys, although its use with large sample sizes is impractical (Zarlenga and La Rosa, 2000). The technique has also recently been applied in identifying species and typing of different geographical strains (Šnábel *et al*., 2001; La Rosa *et al*., 2003).

Genotype-specific DNA repetitive probes have also been used for species identification (Dame *et al*., 1987; La Rosa *et al*., 1994).

Today, DNA-based PCR techniques are the method of choice, displacing in many cases allozyme analysis and other techniques. Diagnosis based on molecular characters tends to minimize the subjectivity that is inherent in biological and morphological characters (Zarlenga and La Rosa, 2000). Methods utilizing PCR technology can be divided into those involving nonspecific primers and those involving genotype-specific primers. Since 1993, the International Commission on Trichinellosis (ICT) has recommended *Trichinella* isolates to be characterized by genetic means rather than by other methods (Lichtenfels *et al*., 1994).

5.6.3.1 Random amplified polymorphic DNA-PCR

The random amplified polymorphic DNA-PCR (RAPD-PCR) method requires a single, short, non-specific primer that is used under nonspecific amplification conditions. The fingerprint pattern obtained is compared with results of reference strains. The method has been utilized to identify the species of a single *Trichinella* muscle larva (Bandi *et al*., 1993b). Different RAPD primer sets have been evaluated in *Trichinella* species detection (Dupouy-Camet *et al*., 1994; Rodríguez *et al*., 1996; Bandi *et al*., 1995). By testing 40 isolates from eight taxa and

different primers, Bandi and others (Bandi *et al*., 1995) developed a method appropriate for routine use in species detection. Several epidemiological studies have been performed using RAPD-PCR in *Trichinella* species detection (Pozio *et al*., 1995; Rodríguez *et al*., 1996; Pozio *et al*., 1998; Wacker *et al*., 1999; Sukura *et al*., 2001; Van der Giessen *et al*., 2001).

The strengths of RAPD-PCR are its speed, simplicity, and sensitivity. Its most prominent weakness is poor reproducibility due to variation in sample DNA quality, contaminations, and reaction conditions (Zarlenga and La Rosa, 2000). The technique needs to be re-optimized when moved from one laboratory to another.

5.6.3.2 Multiplex PCR

Based on observations of variation in the expansion segment V (ESV) region in ribosomal DNA, a multiplex-PCR test was developed (Zarlenga *et al*., 1999; Zarlenga and La Rosa, 2000). It is capable of distinguishing eight *Trichinella* species or genotypes (Pozio *et al*., 1999a; Zarlenga *et al*., 1999). By combining five primers in a single PCR reaction, each genotype can be recognized by a specific amplification profile. This multiplex PCR technique can also be performed on DNA of an individual larva (Zarlenga *et al*., 1999). On the other hand, if the method is applied to samples of pooled larval DNA, it can lead to ambiguous results due to concurrent infection with several *Trichinella* species in the individual host (Zarlenga and Higgins, 2001).

5.6.3.3 Other molecular methods

Reverse line blot hybridization assay (RLB) is based on variations in DNA sequences between 5S ribosomal DNA genes within the genus *Trichinella*. The amplified 5S rRNA region is analyzed using a cleavage fragment length polymorphism assay. Genotype-specific oligonucleotides immobilized on a membrane are hybridized with biotin-labeled PCR products. One PCR-based assay was found to simultaneously identify eight different *Trichinella* genotypes. The method could identify single larvae, it was 10-fold more sensitive than agarose gel analysis, and it was capable of identifying arbitrarily mixed DNA samples of two different *Trichinella* species (Rombout *et al*., 2001).

A single-strand conformational polymorphism technique (SSCP) has also been used to differentiate genotypes of *Trichinella* (Gasser *et al*., 1998). The method offers the advantage of distinguishing species and intraspecies variation, but it is laborious and needs radioisotope markers for visualization of the results (Zarlenga and La Rosa, 2000).

5.7 Epidemiology and distribution

The genus *Trichinella* has a worldwide distribution. Some of its species occur in large geographical areas on several continents, whereas others have more limited distribution patterns. Distribution may be affected by, for instance, genetic adaptation to certain host species and ability of muscle larvae to tolerate environmental conditions in carrions.

5.7.1 Domestic and sylvatic cycles

The epidemiology of *Trichinella* species has traditionally been described as having either a domestic or a sylvatic cycle. The sylvatic cycle occurs in wildlife without contact with human habitation, whilst the domestic cycle acts in human settlements and involves domestic animals like pigs and horses. Synanthropic animals, e.g. cats and rats, live near human habitation and may have a role in transmitting the infection to pigs. Humans can become infected from both domestic and sylvatic cycles. The cycles vary in relation to the host and parasite species involved in different parts of the world (Campbell, 1988; Pozio, 2000; 2001).

The main species involved in the domestic cycle is *T. spiralis*. All of the other *Trichinella* species seem to act mainly in the sylvatic cycle and vary according to the region (Pozio, 2000). The sylvatic and domestic cycles do not exclude each other; they can exist in parallel and even overlap. The domestic species *T. spiralis* has been detected in wildlife, and sylvatic *Trichinella* species have been reported in domestic animals. In Sweden and Germany, *T. spiralis* has been found in wild red foxes (Wacker *et al*., 1999; Pozio *et al*., 2004b). In Canada, it has been observed in wild red foxes, coyotes (*Canis latrans*), and polar bears (Dame *et al*., 1987; Appleyard *et al*., 1998), and in USA in bobcats (*Felis rufus*), black bears (*Ursus americanus*), skunks (*Mephitis nigra*), raccoons (*Procyon lotor*), and opossums (*Didelphis virginiana*) (Dame *et al*., 1987; Murrell *et al*., 1987). Some freezing-resistant *Trichinella* species were detected in pigs in Sweden already in 1948, indicating an infection by a sylvatic *Trichinella* species (Brandt and Hülphers, 1948). In Estonia, *T. nativa* has been identified in sylvatic wild boars (Pozio and Kapel, 1999) and *T. britovi* in a domestic pig (Pozio *et al*., 1995; Järvis *et al*., 2002). In Finland, *T. pseudospiralis* was reported in farmed wild boar (Sukura *et al*., 2001).

The existence of multiple infections of sibling *Trichinella* species was experimentally shown in mice with *T. spiralis* and *T. nativa* (Zarlenga, 1994). Hosts infected naturally by two different species have also been reported. The infection can be a mixture of domestic and sylvatic parasite species. In Estonia, a raccoon dog was demonstrated to be infected by *T. nativa* and *T. britovi* (Pozio *et al*., 1995), and in Spain, wild boars were infected simultaneously with *T. spiralis* and *T. britovi* (Pozio *et al*., 1997).

5.7.2 Climatic effects

The ability of muscle larvae to survive in frozen muscles depends on the host species and *Trichinella* species involved (Pozio *et al*., 1992a). The occurrence of some *Trichinella* species and genotypes seems to correlate with environmental temperature (Pozio *et al*., 1996; Pozio *et al*., 1998; Murrell *et al*., 2000). *Trichinella britovi* and *T. nativa* have a wide distribution in the northern hemisphere. The northern isotherm limit of *T. britovi* is proposed to be –6°C in January (Pozio *et al*., 1996; Pozio *et al*., 1998). *T. nativa*, in turn, is proposed to have a southern isotherm limit of –4°C or –5°C in January (Pozio *et al*., 1998; Pozio, 2000). In contrast to the northern species, muscle larvae of *T. nelsoni* are tolerant to relatively high temperatures, presumably showing an adaptation to warm climates (Boev and Sokolova, 1981; Murrell *et al*., 2000).

5.7.3 Other environmental factors

Trichinella spiralis occurs ubiquitously. This may not be primary due to a wide range of host species but to worldwide distribution of domestic pigs and brown rats (Murrell *et al*., 2000). Human activity may also have contributed to the parasite distribution.

The ability to survive in decaying cadavers is apparently essential in the epidemiology of *Trichinella* species. In both domestic and sylvatic cycles, the infection is presumably often transmitted via decomposed meat (Campbell, 1988). The tolerance to degradation processes has been examined in mouse and fox tissues. The larvae maintained their infective capacity better in rodent than in carnivore hosts. The larvae resisted putrefaction related to the age of the infection, with resistance being higher with older infections. The parasite species influenced preservation ability as well, the most resistant being *T. britovi* and *T. nelsoni*. Among *T. spiralis*, *T. nativa*, *T. murrelli*, T6, *T. britovi*, *T. nelsoni*, and *T. pseudospiralis*, the least resistant to decaying was *T. pseudospiralis* (von Köller *et al*., 2001). *Trichinella pseudospiralis* has previously also been reported to be only weakly resistant to putrefaction (Stewart *et al*., 1990). *Trichinella spiralis* muscle larvae have been demonstrated to survive in buried carcasses. The larvae remained infective in pig muscles buried at depths of 30-100 cm and at temperatures ranging between 4°C and 13°C for three months (Jovic *et al*., 2001).

Trichinella infection can also be transmitted by feces (Zimmermann *et al*., 1959; Robinson and Olsen, 1960). In practice, this route of transmission plays a minor role in epidemiology since parasites are expelled only for short periods of time after infection and survive two to four hours at most (Robinson and Olsen, 1960).

5.7.4 Host diversity

Many of the *Trichinella* species have a wide range of host animals. In 1961, Madsen counted 48 different mammalian host species of *Trichinella* around the world (Madsen, 1961). In 1988, the parasites had been detected in approximately 150 different mammalian hosts (Campbell, 1988). A recent list presents 70 mammalian hosts or host groups in which infection have been confirmed by parasite isolation or serology. The total number of host species is actually much higher since this list includes many large taxonomic host groups like monkeys, bats, voles, and mice (Kapel, 2000). *Trichinella pseudospiralis* is a good example of a species with a wide host range and an extensive geographical distribution, undoubtedly the result of its migrant bird hosts.

5.7.5 Sources of domestic infections

Domestic pigs can become infected with *Trichinella* after eating infected meat scraps from rodent or other animal carcasses, or consuming contaminated feed. Cannibalism, predation, scavenging and, in theory, even eating infected feces play a role. Traditionally, rats have been considered the main infective source of pigs. Rats may be involved in maintaining an infection on a single farm or they may carry the infection from one pig farm to another (Schad *et al*., 1987; Smith and Kay, 1987). Rats from dumps have been shown to frequently be

infected with *Trichinella* (Mikkonen *et al*., 2005). Other wild animals living close to pig farms may also be involved in the infection cycle, not only by transmitting the infection to pigs but also by transmitting it from pigs (Murrell *et al*, 1987; Leiby *et al*., 1988). In Finland, the first detected cases of trichinellosis in pigs were associated with high numbers of rats inside and near barns (Rislakki, 1956a). However, contradictory results have also been presented on the role of rats. In Croatia, a survey of 60 pig farms with or without *Trichinella* infection showed that rats were infected only when pigs were too, and the authors concluded that the rats had become infected via pork scraps spread in the environment (Stojcevic *et al*., 2004). Risk factors for *Trichinella* infection in domestic pigs were analyzed on pig farms in northeastern USA. Statistically significant risk factors were exposure to live wildlife and wildlife carcasses. Other factors with remarkable relative risk were waste-feeding of the pigs and evidence of rodent infestation, but these were not statistically significant (Gamble *et al*., 1999).

To explain *Trichinella* transmission to horses, two hypotheses have been proposed: 1) grazing in pastures contaminated with infected rodent carcasses or pork scraps and 2) feeding with infected flesh from pigs or wild carnivores. However, the infection routes remain unclear (Pozio, 2001). In Serbia, a recent epidemiological study did reveal that owners had fed their horses with animal proteins and kitchen waste prior to selling them (Murrell *et al*., 2004). According to some reports, herbivores in the Arctic and elsewhere will eat meat voluntarily under certain circumstances (Madsen, 1961; Murrell *et al*., 2004).

5.7.6 Epidemiology in Finland

The earliest epidemiological data on trichinellosis in Finland are from the 1800s. An autopsy survey of 1000 Finnish human corpses in 1860s revealed no trichinellosis infections. Unfortunately, Sievers does not mention the method used by Hjelt (Hjelt, 1872, cited by Sievers, 1906; Rislakki, 1956a). The first three human cases reported were members of a family that lived around 1890 in Ruokolahti in southeast Finland (Sievers, 1906; Rislakki, 1956a; 1956b). The source of their infection was believed to be a ham imported from St. Petersburg, Russia. Another autopsy survey was carried out 100 years after the first one. All 100 examined Finnish corpses were negative for trichinellosis. The subjects were men and women with a mean age of 62 years. The study was performed by examining 24 compressed muscle samples from the diaphragm and intercostals with a microscope, and another 24 samples with a trichinoscope (Järvinen *et al*., 1961).

In 1963, an employee of the Helsinki Zoo became acutely ill, manifesting a range of symptoms for years. A diagnosis of trichinellosis was ultimately set in 1967 (Mäkelä, 1970). The subject was subsequently discovered to have eaten some meat from a wild boar raised and slaughtered at the zoo. According to Valtonen (1979), there was another case of trichinellosis in 1976. In 1977, three persons got trichinellosis by eating bear meat in Lapland, northern Finland (Salmi, 1978). Serum samples (n=550) from Lappish families from Inari were tested with two immunological methods in 1970s. Some reactivity to *T. spiralis* antigen was shown, but the author believed it to be nonspecific. These results were similar to those in healthy Swedes (Ljungström, 1979). Thus, human trichinellosis has been very rare in Finland, with only eight cases reported in total.

The examination of Finnish pork for trichinellosis began in 1867. The activity expanded and became nationwide in 1923 (Rislakki, 1956a). No domestic trichinella infections were found in Finnish pigs in 1900-1909, but imported pork from USA was trichinella-positive several times (Rislakki, 1956a). In 1919, trichinellosis was detected in a slaughtered pig in Helsinki; the animal had been imported alive from Russia (Rislakki, 1956a). Again, in 1940, no trichinellosis had been detected in Finnish pigs, but imported pork was infected (Tarnaala, 1940).

The first domestic case reported in a Finnish pig was in 1954. The pig originated from Luvia, southwest Finland. According to the author, the pig had not been fed with carcasses, but rats were plentiful in the piggery (Salmi, 1954; Rislakki, 1956a). Between 1954 and 1960, 16 pigs were trichinella-positive in meat inspection countrywide (Järvinen *et al*., 1961).

Until the 1980s, *Trichinella* infections detected at meat inspection of pigs were rare, typically affecting only a few pigs a year, if any (Salmi, 1958; 1959; Hirvelä-Koski *et al*., 1985; Oivanen and Oksanen, 1994). At the beginning of the 1980s, the number of positive pigs began to increase (Figure 2). In 1983, there were 3.7 positive pigs per million slaughtered. Simultaneously with the increasing number of detected infections in pigs, the detection method has improved. Before 1982, the *Trichinella* examination for meat inspection was done using the trichinoscopy method, but since then the pooled-sample artificial digestion method has replaced it in the largest slaughterhouses. However, the improved sensitivity of detection alone does not explain the increase in detected prevalence. The detection limit of the trichinoscopy method is estimated to be 3 lpg, while the limit of the pooled sample digestion method with 1.0g samples is 1 lpg (SCVPH, 2001). In 1983-1992, a total of 240 infections were detected in pigs. The infection intensity was <1 lpg in 34%, 1-10 lpg in 27%, 10-100 lpg in 27%, and ≥100 lpg in 11% of cases (Hirvelä-Koski *et al*., 1985; Oivanen and Oksanen, 1994). This means that at least 38% of cases, *i.e.* 91 pigs (>10 lpg), would likely have been detected as trichinella-positive at meat inspection by trichinoscopy. The number of infected animals, an average of nine per year, is notably more than it was in previous decades in Finland (Figure 2). During recent years (1995-2003), the proportion of infected pigs has been on average 17 per million examined (range 0-103 per million examined, in total 0-217 positive pigs per year) and the prevalence 0.002%. Trichinella-positive pigs have originated from several farms, with typically only a few positive animals per farm. The number of infected farms has followed the number of infected pigs, being on average 3 (range 0-19) per year (Oivanen and Oksanen, 1994; MAF, 1999; 2001b; 2003; 2004a; 2004b).

From 1990 to 2003, a total of 30 282 horses have been examined for trichinellosis in Finland, with no positive isolations. The examination has been done at meat inspection by either trichinoscopy or artificial digestion, according to national legislation. (MAF, 1993; Oivanen and Oksanen, 1994; EELA, 1994; MAF, 1999; 2001b; 2003; 2004a; 2004b) Since September 2002, the examination of horses has been done by only artificial digestion of 10-g samples (MAF, 2002).

At the Helsinki Zoo, situated on a small island, three polar bears (*Thalarctos maritimus*) have been found to be infected by *Trichinella*; the first case was detected in 1923 and the other two in 1941. In the first bear, the source was unknown, but in the two cases that

followed it was assumed to be Finnish dog meat. The bears had been fed with dog meat during the war time (Hindersson, 1942). In 1965, 76 rats were caught at the zoo and examined. The detected prevalence of *Trichinella* was 12% (Tiainen, 1966).

A survey of wild red foxes revealed a prevalence of 16% (n=38) in 1955-1956. The simultaneous prevalence in brown rats was 2.5% (n=40, Rislakki, 1956a). Wild foxes were also studied in 1963-1964, when the detected prevalence of trichinellosis was 3.8% (n=105) in the whole country and 4.5% (n=89) in the southern part of Finland (Freeman, 1964) (Figure 2).

In the 1980s, trichinellosis in pigs was mostly found in southwest Finland. The prevalence of *Trichinella* infections in wildlife originating from the pig infection area was compared with wildlife originating from other parts of the country. The prevalence in foxes was 43% vs. 29% (n=7 in both groups), in raccoon dogs 25% vs. 9.0% (n=24 and n=11), and altogether in wild carnivores 28% vs. 3.6% (n=50 and 84, respectively). The difference between the prevalence in the animal groups was significant, but the total number of examined animals was small. The infection was also common in farmed red foxes (12%, n=94) and cats from the infection area (41%, n=17) (Hirvelä-Koski *et al*., 1985).

Between 1985 and 1992, the prevalence in foxes was on average 57% (n=68), in southern Finland being 81% and in northern Finland only 5% (Oivanen and Oksanen, 1994). The prevalence observed in raccoon dogs was 50% (n=134) (Mikkonen *et al*., 1995). In 1996-1999, the prevalence in foxes was 35% (n=255) and in raccoon dogs 41% (n=211) (MAF, 1999). The most recent data (2000-2003), indicate a prevalence of 17% (n=349) in foxes and 37% (n=180) in raccoon dogs (MAF, 2001b; 2003; 2004a; 2004b).

In wild boars, the prevalence of trichinellosis in 1985-1992 was 1.3% (n=155). These animals (the majority being farmed not wild) were tested in meat inspection mostly using the trichinoscopy method (Oivanen and Oksanen, 1994). The number of annually slaughtered wild boars has been on the rise because farming has expanded. In 1995-1999 the prevalence in farmed wild boars was 0.7% (n=2265) and in 2000-2003 0.12% (n=4170). Interestingly, the prevalence of trichinellosis in sylvatic wild boars in 2000-2003 was clearly higher than in farmed ones, 33%, but the number of tested animals was only 6 (Sukura *et al*., 2001; MAF, 2001b; 2003; 2004a; 2004b).

The epidemiological situation among lynx (*Lynx lynx*) was described in 1998 by Oksanen *et al*. Of the 327 examined lynx in 1989-1994, the prevalence of trichinellosis was 40%. The authors detected clear geographical differences in the prevalence; the proportion of infected lynx was 5% in the north, 37% in the southwest and 44% in the southeast part of the country. The infection density of positive animals was rather low, with a mean of 3.5 lpg and a median of 1.0 lpg. In 2000-2003, the prevalence was still high: 43% positive among 214 tested (MAF, 2001b; 2003; 2004a; 2004b).

Brown rats from dumps have also been examined in Finland. A total of 767 rats originating from 13 dumps (564 sampled in 1994 from 12 dumps, and 203 in 2000 from one dump) showed a prevalence of 19% (Mikkonen *et al*., 2005).

Based on the results of two host individuals, a domestic pig and a wild red fox, *T. spiralis* and *T. nativa* at least were known to exist in Finland (Pozio, 1995). A few years ago, *T. pseudospiralis* was also reported in Finland (Sukura *et al*., 2001; II, III).

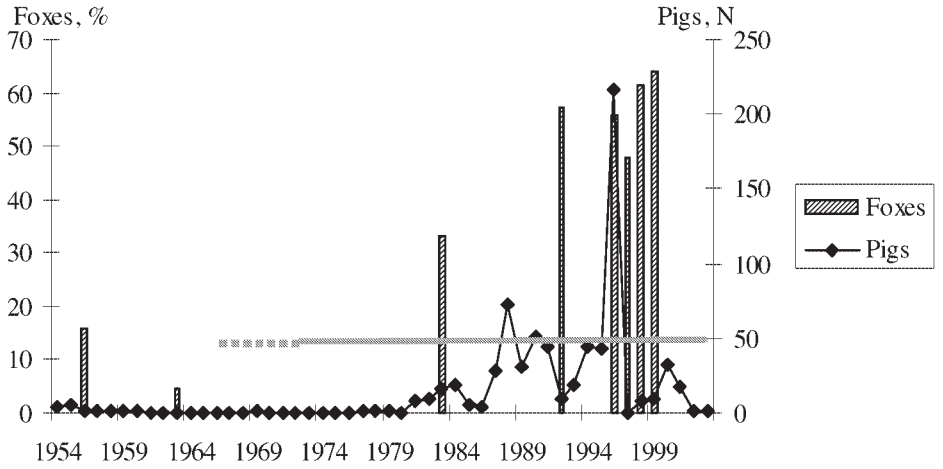

Figure 2. Trichinellosis in pigs (number of annually detected cases 1954-2003), prevalence in foxes and dispersion of raccoon dog to Finland (grey horizontal line).

5.7.7 Epidemiology in other European countries

In contrast to Finland, outbreaks of human trichinellosis have been detected in Sweden a few times. In the 1970s, the yearly average number of the detected cases was 2.1, which comprised 0.00003% of the Swedish population (Ronéus and Christensson, 1979). An epidemic involving 15 persons occurred in 1969. Although the source of infection was not identified, pork was suspected. Between 1917 and 1969, nine epidemics (altogether 489 cases) and a few sporadic cases were reported. Sources of the epidemics were bear meat and pork (Odelram, 1973). Infections in pigs have been detected at meat inspection as early as in 1867, when the prevalence ranged from 0.38% to 2.16%. In the 20th century, the prevalence in pigs has steadily decreased, being 0.08% in 1915, 0.002% in the 1940s (15 positive out of 600 000 slaughtered), and 0.0003% in 1960. Ekstam noticed that the number of positive pigs varied in 10-year periods. Fox and pig carcasses were thought to be the main source for the infections in pigs. The detected prevalence in foxes was 14% and in badgers 2%. (Ekstam, 1964a; 1964b). In the 1970s, *Trichinella* incidence in pigs decreased to 0.00018% (52 positive out of 29 300 000 slaughtered in 1970-1977). The prevalence of trichinellosis in the fox population declined from an average of 20% in 1979 (Ronéus and Christensson, 1979) to 10% in 1994 (Christensson, 1994). However, the prevalence in 1979 varied considerably in different parts of the country, ranging from 6% to 48% in the mainland. The prevalence of trichinellosis in foxes did not show a clear difference between southern and northern parts of Sweden (Ronéus and Christensson, 1979).

Within the past few decades, no human cases and very few infections in pigs have been reported in Sweden (EC, 2003; 2005). Four *Trichinella* species have been identified: *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis* (Christensson, 1994; Christensson and Pozio, 2004; Pozio *et al.*, 2004b).

In Estonia, the average number of annually reported human infections in the past decade is seven. However, in 1993, 43 persons acquired an infection. Most of these infections originated from game meat. Only three persons were infected by pork (Järvis *et al*., 2001). The prevalence of trichinellosis in wild boars is about 1%, in red foxes 42-44%, in raccoon dogs 50-53%, and in lynx 38-47% (Pozio *et al*., 1998; Järvis *et al*., 2001). *Trichinella spiralis*, *T. britovi*, and *T. nativa* have been reported (Pozio *et al*., 1995).

In Latvia, trichinellosis in wild boars is widespread. In 1976-1998, the prevalence ranged from 1.3% to 3.2% (Keidans, 1999, cited by Järvis *et al*., 2001).

In Lithuania, the number of human trichinellosis cases has risen between 1969 and 1995. In 1969-1992, the number of annually reported cases ranged from 6 to 819. The annual number of epidemics was greatest (n=79) in 1992. During 1982-1992 nine fatal cases occurred. In 1995, the morbidity index was 10.8 per 100 000 inhabitants. Serological prevalence in the population was high, 23-30% in healthy persons. The source of the infections was mainly pork or wild boar meat. The prevalence in animals increased simultaneously with human infections. In 1992, the prevalence in pigs was 0.05%, and in wild boars 1.2%. The prevalence in foxes was 18%, in lynx 23.5%, in wild dogs 18%, and in domestic dogs 8.9% (Ročkiene, 1994; Ročkiene and Ročka, 1997).

In Norway, human trichinellosis has not been detected since 1953. Before that epidemic involving five persons, six epidemics, affecting a total of 711 patients, had been reported since 1881. In pigs, the infections have been rare. In the 1950s and 1960s, there was a peak in the number of positive pigs detected (37 positive farms in 1950), but since 1981 no further *Trichinella* infections have been detected in pigs (Skjerve, 1993; EC, 2003; 2005).). In wildlife, the prevalence of trichinellosis has been highest in foxes, 25%, but infections have also been detected in badgers, pine martens (*Martes martes*), minks (*Mustela vison*), and wolverines (*Gulo gulo*) (Stuve and Holt, 1993).

Unlike in the other Scandinavian and Baltic countries, *Trichinella* infections in animals are remarkably rare in Denmark. In a screening of red foxes in 1995-1998, only three animals of the 6141 examined were infected (0.0005%). According to Enemark and others (2000), the prevalence of *Trichinella* infections among Danish foxes has been less than 0.1% for decades. *Trichinella* examination has been compulsory for pigs in meat inspection since 1930. Since then, no positive pigs have been detected. For more than 50 years, no reports have been made of any autochthonous human infections (Enemark *et al*., 2000).

In Central Europe, *Trichinella* infections seem to exist in wildlife with low prevalence. In Germany, a survey of 7103 foxes from the Brandenburg region revealed five infected individ-

uals (0.07%). However, of 3295 tested fox sera, 7.7% were serologically positive (Wacker *et al*., 1999). In Switzerland, *Trichinella* larvae were detected in 0.01% of examined foxes, but serologically the prevalence was 12.6% (Gottstein *et al*., 1997). In Belgium, the seroprevalence of trichinellosis in foxes was 2.7%, while with the artificial digestion method no positive foxes were identified (Vercammen *et al*., 2002). In the Netherlands, the prevalence in foxes was 5.1% by the artificial digestion method. In wild boar, the seroprevalence was 6.8%, and this has shown an increasing trend (van der Giessen *et al*., 2001). *Trichinella* species detected in Germany are *T. spiralis* in wild boars, foxes and pigs, and *T. britovi* in foxes (Pozio *et al*., 2000). In the Netherlands, the detected species are *T. spiralis* in wild boars and *T. britovi* in foxes (van der Giessen *et al*., 2001).

In contrast to continental Europe, trichinellosis was not detected in wildlife in Great Britain. A recent survey of 587 foxes revealed no infected animals (Smith *et al*., 2003). From Ireland, there is a report of three *Trichinella* positive foxhounds of the five actively used for fox hunting (O'Rourke and Verling, 1972). In Central and Southern Europe, *T. spiralis*, *T. britov*i, and *T. pseudospiralis* have been detected in wildlife (Pozio, 2000).

According to annual reports of zoonotic agents in the EU, domestic trichinellosis has been detected in Finland, France, Italy, the Netherlands, and Spain in 1996-2003. Wildlife trichinellosis was reported for the same period in Finland, Austria, France, Germany, Ireland, Italy, the Netherlands, Norway, Spain, and Sweden. In 2003, infections in pigs were detected in Finland, Germany, and Spain, and infections in wild boars in Finland, France, Germany, Italy, the Netherlands, Spain, and Sweden. The differences in total numbers of examined animals in different member states were large. Domestic and sylvatic infections were the most prevalent in Finland and Spain in 1996-2003 (EC, 2002a; 2003; 2005).

Besides in the Baltic countries, *T. spiralis* is known to occur in domestic animals and in the domestic cycle of the following new EU member countries: Hungary, Poland, and the Slovak Republic. East-European countries such as Bulgaria, Byelorussia, Romania, Serbia, and Ukraine, have the same situation. Wildlife trichinellosis is even more prevalent, and *T. spiralis* has been detected in the sylvatic cycle in the same countries. Human infections have been reported annually in all these countries except Hungary and Ukraine (Pozio, 2001).

In the European part of Russia, domestic and sylvatic trichinellosis has been detected frequently. In North-West Russia, in the Tvier and Smolensk regions, the prevalence in wolves (*Canis lupus*) was as high as 97.3% (n=73). Wolf and dog carcasses seemed to represent important feed sources for wolves, maintaining the *Trichinella* infection in the population. The prevalence in foxes was 48% and in domestic dogs 7.7%. The predominant species was *T. nativa*, but *T. britovi* was also found (Casulli *et al*., 2001; Pozio *et al*., 2001c). In Russia, human infections have been reported annually (Pozio, 2001).

Since 1975, at least 14 human trichinellosis epidemics have been reported in the EU as a result of consuming raw or inadequately cooked horse meat. All of these outbreaks have taken place in Italy and France, where consumers have the raw "blue" meat culinary habit. Alto-

gether 3326 patients have been reported (Boireau *et al*., 2000; Pozio, 2001). In 1975-1999, the estimated incidence of trichinellosis in horses was 3.5 per one million slaughtered in EU. Only 27 infected horses had been reported worldwide. The infected horses have been imported from endemic countries, including Mexico, Poland, Serbia, and Romania (Pozio, 2001; Pozio *et al*., 2001d).

In Germany, the number of human infections reported has ranged from 0 to 10 per year in 1990-1997. In 1998-1999, two separate epidemics occurred, involving 52 people (Pozio *et al*., 2000; Nöckler *et al*., 2001). In Great Britain, human cases were not reported in 1969-1999. However, in 1999, an epidemic of affecting eight persons occurred. The pork salami responsible for the infections had been privately brought from Serbia, the former Yugoslavia (Milne *et al*., 2001).

In Spain, human trichinellosis remains a public health threat. Epidemics of trichinellosis are detected and reported regularly, 2-3 per year. Most of these are caused by *T. spiralis*, but recently *T. britovi* has also been implicated. In 2000, 38 people became infected, and in 2001-2002, 26 cases were diagnosed (Cortés-Blanco *et al*., 2002; Gomez-Garcia *et al*., 2003).

According to annual reports of zoonotic agents in the EU, human trichinellosis was detected in Austria (number of positive cases =10), France (140), Germany (106), Italy (99), Spain (307), the Netherlands (22), United Kingdom (2), and Norway (4) between 1995 and 2003. In all of the countries, except in France, Italy and Spain, the infections were mostly imported. The total number of human infections acquired in the EU has ranged between 48 and 67 per year in 1999-2003 (EC, 2002a; 2003; 2005).

5.7.8 Global epidemiology

In USA, trichinellosis in pigs is not tested routinely. In a survey in two northeastern states in 1990s, the prevalence in pigs was 0.37% (n=4078), and the proportion of positive farms was 6.4% (n=156). The farms were not chosen randomly but selected to represent different feeding types. The prevalence of trichinellosis in pigs in the US has shown a declining trend (Gamble *et al*., 1999). In 1991-1995, 230 human cases were reported, three of them leading to death. The number of annually reported human cases has decreased since 1947; in the 1990s it was less than 50 cases per year. The most common source of infections has been pork, but the proportion of wild game is on the rise (Moorhead *et al*., 1999). In the temperate regions of North America, *T. spiralis*, *T. murrelli*, T6, and *T. pseudospiralis* have been detected (Gamble *et al*., 1999).

In Canada, *Trichinella* infections in pigs have been reported in limited areas, and farmed wild boars are infected only sporadically. A survey of 14 408 sows showed no positive results (Gajadhar *et al*., 1997; Appleyard *et al*., 2002). However, *Trichinella* infection is prevalent in wildlife. Human outbreaks have occurred due to consumption of wild game (Dick *et al*., 1986; Schellenberg *et al*., 2003) or very rarely farmed wild boar (Greenblom *et al*., 1997). In northern Canada, human trichinellosis is prevalent due to consumption of raw walrus meat and some

traditional undercooked meat dishes. The species responsible has been *T. nativa* (Forbes *et al*., 2003b; Leclair *et al*., 2003).

Human trichinellosis is present in Mexico. In 1972-1973, an autopsy survey indicated that 4.2-7.6% of corpses had larvae in the diaphragm (Martinez-Marañón *et al*., 1974, cited by de-la-Rosa *et al*., 1998). In the 1990s, a survey showed antibody prevalence in 1.0-1.9% of the population. Risk factors were female gender and ingestion of pork sausage (de-la-Rosa *et al*., 1998). In South America, numerous *Trichinella* outbreaks and human infections have been reported in Chile and Argentina. The parasites have also spread to other nearby countries. However, detailed surveys and data are unavailable (Ortega-Pierres *et al*., 2000).

Human trichinellosis also occurs in southeast Asia. Most of the outbreaks have been reported in China and Thailand. In China, 544 outbreaks have been reported in 1964-1998, affecting over 25 000 people. Of 4719 people tested, 12% were infected. The reported prevalence in pigs and dogs, which are also eaten, has been 10% and 27%, respectively. In Thailand, 120 outbreaks have occurred in 1962-1999, involving 6700 patients. The detected prevalence in domestic pigs has been 0.02%, in hilltribe pigs 11%, and in dogs 49% (Takahashi *et al*., 2000).

Evaluation of the trichinellosis situation worldwide is difficult because insufficient epidemiological data are available for many countries. However, the disease is emerging or re-emerging in many areas, and thus, efforts should be directed at controlling the infection (Dupouy-Camet, 2000).

5.8 Control of trichinellosis

In Finland, the examination of domestic pork began in 1867 in Helsinki. In the same year, importing pork was prohibited. The ban was later cancelled, but all imported pork had to be examined for trichinellosis. The examination of pork expanded from Helsinki into other large cities but did not become obligatory nationwide until the first act for meat inspection was passed in 1923 (Rislakki, 1956a). Since no infected pigs had been detected for decades, examination was discontinued in 1948-1954, except in exporting slaughterhouses. The examination was, however, reinstated after the first new case of trichinellosis was detected in a Finnish pig in 1954 (Salmi, 1954; Rislakki, 1956a). Since then, all slaughtered pigs have been examined in meat inspection. From 1990 onwards, all slaughtered horses have also been examined (MAF, 2004b).

Trichinellosis is an infection that is monitored and controlled based on legislation of animal diseases in Finland. When infected animals are detected at meat inspection, they are traced back to the original farms and their infection routes mapped. Kitchen leftovers may not be fed to pigs unheated (MAF, 1980). All meat intended for the market is to be inspected. Meat inspection of domestic and wild mammals that are potential hosts for *Trichinella* always includes an examination for trichinellosis. In the case of a positive result, the whole carcass is condemned. Infection is confirmed at the EELA (MAF, 2001a). According to EC regulation on animal by-products, all condemned and dead animal carcasses shall be destroyed or

buried in the ground (EC, 2002b). Human trichinellosis is a notifiable disease in Finland. Data on annual occurrence in animals and humans are reported to the EU (MAF, 2004b).

In the EU, many member countries require *Trichinella* inspection for every slaughtered pig, but some require it only for pork traded to other member countries (Nöckler *et al*., 2000). In countries where meat inspection for *Trichinella* is not mandatory, other methods to control infection in pigs and humans are used. For example, in the US, consumers are advised to cook or freeze pork properly at home (Bruschi and Murrell, 2002). Another relevant control is good farm management practices, including rodent control and avoidance of feeding waste to pigs (Gajadhar and Gamble, 2000).

5.8.1 Trends and challenges of trichinellosis control

The meat industry continually seeks means to reduce the costs of meat inspection and to hasten the passage of carcasses in slaughterhouses. In countries and areas where porcine trichinellosis is virtually nonexistent, the industry is often willing to give up the individual testing of pig carcasses. The EU is currently preparing legislation to set aside testing on *Trichinella*-free farms and/or areas, and a similar intent has also emerged in North America. The international organizations OIE and ICT have also stated their opinions on the topic. The requirements for *Trichinella*-free status in the EU will be strict, but some countries or areas with especially low prevalence or no domestic and sylvatic trichinellosis may probably achieve free status. These changes in the international meat markets will influence pork production in many countries (Gajadhar and Gamble, 2000; Nöckler *et al*., 2000; SCVPH, 2001).

Quality assurance requirements in laboratory analysis will eventually impact *Trichinella* diagnostics at meat inspection worldwide. In Canada and some other countries, obligatory proficiency testing of laboratories already exists (Gajadhar and Forbes, 2002; Christensson and Pozio, 2004). The methods used in each laboratory will need to be validated and demands for competence met (Gamble *et al*., 2000).

The increasing interest in organic farming may bring drawbacks and new aspects to the control of *Trichinella*. EU with its new endemic member countries will meet challenges inside the union (Pozio, 2001).

International organizations, such as the Food and Agriculture Organization (FAO), the World Health Organization (WHO), and OIE, are actively involved in work related to improving the safety of animal feedstuffs. They provide guidance on general management to ensure food and feed safety and to control animal diseases globally (Gajadhar and Gamble, 2000; Moreno-López, 2002).

6. AIMS OF THE STUDY

The aims of this dissertation were as follows:

1. To characterize *Trichinella* species in Finnish domestic and sylvatic cycles (I, II).

2. To identify potential reservoirs and vectors for *Trichinella* species occurring in the domestic cycle in Finland (I, II).

3. To compare molecular techniques for identification of *Trichinella* species (I, III).

4. To evaluate infectivity of *Trichinella* species for reindeer (IV).

5. To determine persistence of *T. spiralis* in different environments (V).

7. MATERIALS AND METHODS

7.1 Materials

7.1.1 Animals utilized for descriptive studies

Trichinella-containing muscle samples from pigs, farmed and sylvatic wild boars, and brown bears (*Ursus arctos*) originated from carcasses condemned in meat inspection. These samples were delivered from the slaughterhouses to the National Veterinary and Food Research Institute (EELA) for confirmation of diagnosis. Wildlife for the surveys, comprising red foxes, raccoon dogs, badgers, and wolves was collected by hunters (I, II, III). Samples from lynxes were obtained from the Finnish Game and Fishery Research Institute and the University of Oulu. Brown rats were trapped at dumps in southwestern Finland and delivered to the authors by the University of Turku. Domestic cats (*Felis domesticus*) originated from pig farms in southwest Finland. Serum samples from farmed wild boars were collected by the authors at slaughter (I). All larvae available for identification of *Trichinella* species (II, III) originated from hosts from southern Finland, while the hosts used for the prevalence part of the study (II) represented the whole country.

7.1.2 Experimental animals

Trichinella isolates were maintained in female mice (IV, V) of strain type Naval Medical Research Institute (NMRI), USA. At the time of inoculation the mice weighed approximately 20 g. Mice were bred and housed at the experimental animal unit of EELA, Helsinki, in a separate trichinella infection room, but the stock was purchased from Denmark (M&B a/s, Ry, Denmark).

To study survival of *T. spiralis* in rat carcasses (V), male Wistar rats were used. At the beginning of the experiment, the rats weighed on average 233 g. They were purchased from Harlan Netherland B.V., Horst, the Netherlands, and housed and infected at the experimental animal unit of the Faculty of Veterinary Medicine, University of Helsinki.

Reindeer (*Rangifer tarandus*) for the experimental infection study (IV) were 10-month-old female semi-domesticated calves bred and kept at the Zoological Gardens of the University of Oulu. The study group and the controls were kept separately. Twice weekly, blood samples were drawn from the jugular vein (IV).

Mice and rats were anesthetized with CO_2 and euthanized. At the end of the trial, reindeer were euthanized as in routine slaughter.

The numbers of animals in Studies I–V are shown in Table 1. All experimental protocols were approved by the relevant authorities.[1]

Table 1. Summary of the animals used in Studies I–V.

Animals		Total number of animals	Study
Animals examined at meat inspection*	Domestic pigs	10	II, III
	Farmed wild boars	13	I, II, III
	Sylvatic wild boars	1	II, III
	Brown bears	3	II, III
Hunted/trapped /euthanized animals	Red foxes	177	I, II, III
	Raccoon dogs	200	II, III
	Badgers	6	II
	Wolves	20	II, III
	Wild rats	29	II, III
	Domestic cats	2	II, III
Experimental animals	Laboratory rats	63	V
	Laboratory mice	28 **	IV,V
	Reindeer	10	IV

* Number of animals includes those from which larvae were used for PCR analysis or serum for serology, but not animals used only in the prevalence study.
** In addition to the mice used to infect study groups, four animals on average were used annually to maintain each *Trichinella* strain.

7.1.3 Experimental feeds and conditions in the *Trichinella* survival study

Persistence of *T. spiralis* in different feeds was studied experimentally by storing specimens of *Trichinella*-infected rat carcasses in three different feeds and in conditions that simulated the pasture, i.e. in a shaded box. Temperature was recorded inside the barn, outdoors, and inside the shaded box. The summer of 2001 was warm in Finland. During the study period the weekly average temperature ranged from 18.5 to 25.5°C. Maximum temperature recorded outdoors in the sunshine was 42°C and the minimum 14°C, and the six-week average in the

[1] The County Administrative Board of Uusimaa, Department of Social and Health Affairs, STO62 D. no. 19605 972 89 70/1990.
The County Administrative Board of Uusimaa, KUN583/1997.
The Committee on Animal Experiments of the University of Oulu, 14/97.
The Committee on Animal Experiments of the Faculty of Veterinary Medicine, University of Helsinki, and the National Veterinary and Food Research Institute, April 23, 1998.
The Committee on Animal Experiments of the Faculty of Veterinary Medicine, University of Helsinki, and the National Veterinary and Food Research Institute, January 27, 1999.
The Committee on Animal Experiments of the University of Helsinki, 354/2001.

shaded box was 23°C. The humidity was also recorded in the shaded box. The content of dry matter was noted for each feed but reported as moisture. At the end of the experiment, pH and moisture were monitored in the remaining target rats as well as in a fresh minced rat carcass.

7.1.3.1 Silage

Silage was processed by mixing shredded hay with a formic acid-based preservation solution. The plastic-covered bale of approximately 750 kg was stored outdoors as in ordinary farming. The initial pH of the silage was 5.1, but it dropped to 4.5 during the first week and remayned at this level. The moisture was 71.6-75.1%.

7.1.3.2 Grained barley

Grained dry barley was purchased as ready feed and stored in a lidded 100-l plastic container inside a barn. The pH was 5.8-5.9 and the moisture 12.1-13.5%.

7.1.3.3 Propionic acid-fermented feed

A mixture of barley (30%) and oats (70%) was purchased as ready mixed feed. Seeds were not grained but flattened and mixed with a commercial preservation solution. The pH was constant at 4.8 and the moisture 19.6-21.4%. Propionic acid-fermented feed was stored in a similar plastic container as grain, side by side.

7.1.3.4 Pasture simulation

Pasture outdoor conditions were simulated by storing rat carcasses in a well-ventilated plywood box that was inaccessible to invasion by creatures bigger than ants. This shaded box was placed outdoors.

7.2 Methods

7.2.1 Animal experiments

Both *Trichinella* isolates used in the experimental infections were of Finnish origin. *Trichinella spiralis* was isolated from a Finnish domestic swine from Ypäjä, in southwest Finland, EELA strain no. 970/96, and assigned by the International *Trichinella* Reference Centre (ITRC), Istituto Superiore di Sanità, Rome, the code ISS559 (IV, V). *Trichinella nativa* was isolated from a Finnish wild raccoon dog hunted in Iitti, southeast Finland, EELA 2743/96, ISS558 (IV). Both isolates had been maintained in mice for two generations prior to inoculation in reindeer (IV) and strain ISS559 for eight generations prior to inoculation in rats (V).

Mice used to maintain *Trichinella* isolates (IV, V) were infected by allowing them to eat raw meat containing a known number of infective *Trichinella* larvae. For the use in experimental infections, mice were euthanized 3-5 weeks post-inoculation.

To study experimental infectivity of *Trichinella* for reindeer, 6 of 10 reindeer were infected *per os* (*p.o.*) by gavage with *Trichinella*-infected minced mouse meat (IV). The infection dose was 2500-75 000 larvae of *T. nativa* or 5000 larvae of *T. spiralis*. Four reindeer served as a control group. On day 56 post-inoculation, the inoculated reindeer were euthanized.

To examine persistence of the pathogen in feeds (V), target rats were infected *p.o.* with approximately 300 muscle larvae of *T. spiralis* in minced mouse meat. The rats were killed four weeks post-inoculation. A digestion sample from the left hind leg was taken from each carcass to confirm the initial intensity of the infection. Six rat carcasses were placed in each test environment in polyamide pouches made from 40-denier (den) pantyhose. Recipient rats were used to confirm the infectivity of the larvae found in test environments. These rats were inoculated *p.o.* by a stomach tube with a dose of 300 larvae or less, depending on the recovery of larvae from samples. Recipient rats were euthanized after 6-8 weeks of follow-up, and the intensity of infection was analyzed. To calculate the RCI (Kozar and Kozar, 1965; Dame *et al*., 1987; Bolas-Fernandez and Wakelin, 1989; Pozio *et al*., 1992a), the total number of *Trichinella* in each rat was estimated by multiplying the lpg value by the total weight of the animal and dividing this value by the infection dose.

7.2.2 Artificial digestion

To confirm trichinella-positive meat inspection findings, a total of 5-100 g of muscle tissue was examined for each animal at EELA (I, II). Confirmation was made by using the artificial digestion method. In the case of pigs, wild boars, and bears, the samples were mainly taken from the diaphragm. Wildlife and other animals for the surveys were tested by artificial digestion of samples from the diaphragm, masticatory muscles, or forelimbs (I, II).

From the experimentally infected reindeer, muscle samples were taken from the tongue, heart, diaphragm, *Musculus masseter*, *M. intercostales*, *Musculus longissimus dorsi*, and *M. gastrocnemius*. A 50-g pooled sample was first collected from the diaphragm, tongue, and masticatory muscles and examined with the digestion method. A negative result was confirmed with another 50-g sample. If one or more *Trichinella* larvae were seen in the first or second composite sample, 50-g samples from each of the individual muscle groups were examined separately (IV).

The artificial digestion of all muscle samples (I-V) was performed with pepsin and hydrogen chloride (HCl) according to national meat inspection regulations and the recommendations of the ICT (Gamble *et al*., 2000; MAF, 2002). Intensity of detected infections was determined and presented as lpg in muscles.

7.2.3 PCR techniques

To preserve the larvae for species identification, samples were stored in 70% ethanol in distilled water at –20°C (I-III). Prior to molecular analysis, larvae were rehydrated in a decreasing series of ethanol. Larval DNA for species detection was isolated from single or

pooled samples of larvae for each host animal separately (I-III). The physical condition of the larvae based on their microscopic performance was recorded (III).

RAPD-PCR was used to identify species of *Trichinella* larvae, as described by Bandi and others (Bandi *et al*., 1993; Bandi *et al*., 1995). RAPD-PCR was performed at the Faculty of Veterinary Medicine, University of Helsinki (I) or at the Danish Centre for Experimental Parasitology, Denmark (III). The multiplex PCR technique was applied to identify *Trichinella* larvae at ITRC, Rome, Italy (II, III). The multiplex PCR was performed according to a published protocol (Zarlenga *et al*., 1999), with slight modifications. The sequences of the primers used in PCR analysis are presented in Table 2.

In RAPD-PCR (I, III), reference strains included *T. spiralis* ISS004, *T. nativa* ISS042, *T. britovi* ISS100, and *T. pseudospiralis* ISS013. In multiplex PCR (II, III), larvae of *T. spiralis* ISS003, *T. nativa* ISS010, *T. britovi* ISS002, and *T. pseudospiralis* ISS013 were used as reference strains. The *Trichinella* reference strains were obtained from ITRC, Rome, Italy.

Mixed infections of two species of *Trichinella* were simulated in RAPD-PCR by arbitrary test samples of DNA from *T. spiralis* ISS559 and *T. nativa* ISS558. The mixed samples consisted of the two species in ratios of 1:1, 1:2, and 2:1, and a total of 10 larvae per sample.

Table 2. Primers used in PCR analysis in Studies I, II, and III presented in 5´-3´ order.

Method	Set/ Code	Forward	Reverse
Multiplex	1.	GTTCCATGTGAACAGCAGT	CGAAAACATACGACAACTGC
	2.	GCTACATCCTTTTGATCTGTT	AGACACAATATCAACCACAGTACA
	3.	GCGGAAGGATCATTATCGTGTA	TGGATTACAAAGAAAACCATCACT
	4.	GTGAGCGTAATAAAGGTGCAG	TTCATCACACATCTTCCACTA
	5.	CAATTGAAAACCGCTTAGCGTGTTT	TGATCTGAGGTCGACATTTCC
RAPD	494	AGCGCTGTGAGAAAGATGAAAGAT	

7.2.4 Serological techniques

Seroconversion of wild boars was investigated with ELISA and Western blotting (I). In both techniques, crude antigen of the larval lysate of *T. spiralis* ISS559 was used. For testing reindeer sera (IV), *T. spiralis* excretory-secretory (ES) antigen (Gamble *et al*., 1983) was used in ELISA. The ES antigen was obtained from the Parasite Biology and Epidemiology Laboratory, United States Department of Agriculture, Beltsville, Maryland, USA.

As a secondary antibody, commercial peroxidase-conjugated goat anti-swine antibody was used in wild boars (I) and biotin-labeled rabbit anti-reindeer immunoglobulin (Åsbakk *et al*., 1999) with peroxidase-conjugated streptavidin in reindeer (IV). Hydrogen peroxidase and

tetramethyl benzidine (I) or o-phenylenediamine was added as a substrate (IV). In ELISA, the cut-off absorbance was set at the mean plus two times the standard deviation (SD) of values for wild boars not showing immunoreactive antibodies in immunoblotting (I). In the reindeer assay, individual optical density (OD) values of the inoculated animals were compared with mean value plus SD of the control animals (IV).

Western blotting was performed according to standard methods. Proteins from the crude antigen preparation were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with 4% stacking gel under reducing conditions, and then transferred to a nitrocellulose membrane. Immunodetection was done as with ELISA, but diaminobenzidine was used as a chromogen.

7.2.5 Statistical methods

Logistic models were applied to identify the risk factors for trichinellosis. Results were expressed as observed percentages and odds ratios (OR) with 95% confidence intervals (CI) derived from the model parameter estimates (II).

One-way analysis of variance (ANOVA) was applied to determine host species-specific differences in intensity of infection. Bonferroni's method was used for pairwise comparisons. To normalize data, rank transformation was used when appropriate; a probability (p) value <0.05 was considered statistically significant (II). The kappa coefficient (K) was used to describe and test the degree of agreement between RAPD-PCR and multiplex PCR species identification.

Distribution of different *Trichinella* species was analyzed using the likelihood ratio chi-square test (χ^2) (II). Fisher's exact test was used to determine whether RAPD-PCR was influenced by the preservation of larvae (III) and to evaluate the statistical significance of the distribution of detected *Trichinella* infection in wild boars and their living during the rat invasion (I).

Statistical software comprised the SAS Proprietary Software Release 6.12 (Copyright 1989-1996 by SAS Institute Inc., Cary, North Carolina, USA), Statistix Version 1.0 for Windows 95 (Analytical Software, 1996), and Epi Info Version 5.01b (CDC, Atlanta, Georgia, USA, and WHO, Geneva).

8. RESULTS

8.1 Descriptive studies

8.1.1 Outbreak on a wild boar farm

An outbreak of trichinellosis (I) was described on a wild boar farm in southeast Finland. A rat invasion from a nearby dump had preceded the wild boar infections. A total of nine farmed wild boars of the 25 slaughtered during the observation period were condemned at meat inspection because of trichinellosis. Eight of 14 animals that lived during the rat invasion were trichinella-positive at meat inspection, in contrast to one positive of 11 born after the invasion ($p<0.05$, relative risk (RR) 6.3, $CI^{95\%}$ 1.1-650). The relation between exposure to rats and trichinellosis in wild boars is presented in Table 3. Wild red foxes around the farm (n=19) had a very high prevalence of trichinellosis (74%).

Trichinella spiralis was identified in all of the examined wild boars (n=6). No marks of mixed infections were seen. One of the 13 foxes analyzed was infected by *T. spiralis* and the rest by *T. nativa*.

Three of the seven serum-sampled wild boars showed immunoreactivity in ELISA and Western blotting. Two of these animals were identified as trichinella-positive with a mild infection in meat inspection. In Western blotting, the pattern of immunoreactivity was similar for all positive wild boars, with separate double bands at 100 kD and 3 doublets between 76 kD and 49 kD. The animal with immunoreactive antibodies but negative in meat inspection had the highest absorbance in ELISA.

Table 3. Relationship between wild boars living during the rat invasion (exposed) or afterwards (not exposed) and meat inspection findings (detected infected vs. not detected).

	Infected*	Not infected*	**Total**
Exposed	8	6	**14**
Not exposed	1	10	**11**
Total	**9**	**16**	**25**

* According to meat inspection findings.

8.1.2 Prevalence study

In the study of the whole country (II), the prevalence of trichinellosis varied according to host species and region (north, SW, SE). In the SW, the highest prevalence was observed in lynxes (70%), whereas in the SE, the highest rate was found in foxes (62%) (Figure 3).

The relationship between risk of acquiring trichinellosis and region of the country was evaluated by analyzing data for foxes and bears. The prevalence was higher in foxes in the SW and SE than in the north. Differences between the fox and bear were large in the SW, but not in the north. Region and host species were significant factors according to the logistic model. Region had a greater influence on the risk of trichinellosis in foxes (OR 7.75) than in brown bears (OR 1), and more in southern (SW: OR 14.3; SE: OR 19.4) than in northern Finland (OR 1). When comparing foxes, raccoon dogs, and lynxes between the SW and SE, the lowest prevalence (29%) was observed in raccoon dogs from the SW. The risk of acquiring trichinellosis was significantly different between host species but not between the two southern regions. The infection risk was higher in foxes (OR 2.09) and lynxes (OR 1.92) than in raccoon dogs (OR 1).

The highest intensity of infection was detected in raccoon dogs (median 30 lpg), and the lowest in lynxes (median 0.7 lpg). Differences in infection intensity of positive animals were evaluated by one-way ANOVA for rank-transformed data. Interspecific differences were present ($p<0.0001$), with infection intensity in raccoon dogs being significantly higher than in foxes, bears, and lynxes.

For bears, confirmation of the diagnosis set at meat inspection was done at EELA. For trichinella-positive bears at meat inspection, the mean size of the confirmation sample was 20.0 (range 1.0-100) g. The mean sample size for foxes was 26.0 (range 1.0-100) g, for lynxes 47.0 (range 8.0-100) g, for raccoon dogs 10.0 (range 1.0-100) g, and for wolves 51.0 (range 10.0-100) g. All badger samples were 10 g.

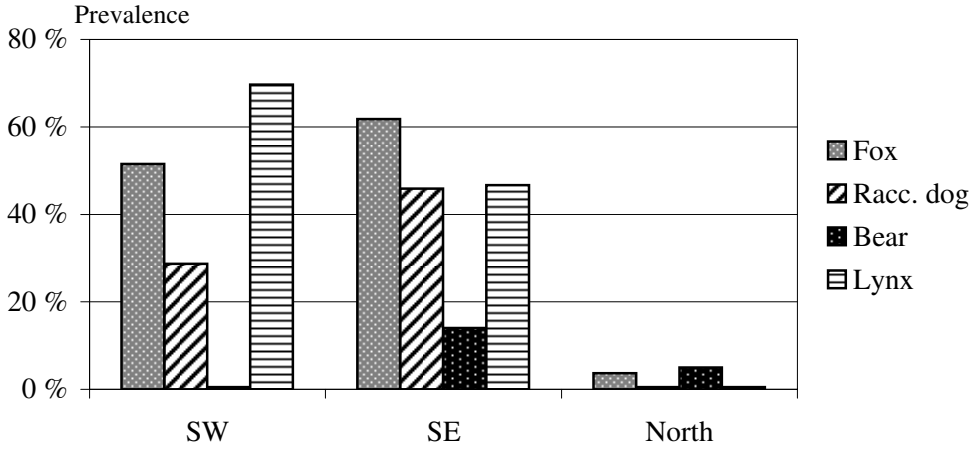

Figure 3. Observed prevalence of trichinellosis in foxes, raccoon dogs, bears, and lynxes by region of the country (SW=southwest, SE=southeast).

8.1.3 *Trichinella* species and hosts

The hosts utilized for species detection had heavy or moderate infections. The median intensity of infections in all host animals was 25.7 lpg. The median infection intensities were as follows: raccoon dogs 64.3 lpg, foxes 15.8 lpg, bears 4.7 lpg, lynxes 2.0 lpg, wolves 53.5 lpg, wild boars 13.0 lpg, rats 30.0 lpg, pigs 13.6 lpg, and cats 39.3 lpg (II, III).

Four species of *Trichinella*, *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis*, were identified. Most of the infected animals (82/87) harbored only one species; *T. spiralis* was detected in 48, *T. nativa* in 28, and *T. pseudospiralis* in 6 animals (II). *Trichinella spiralis* was detected more often in domestic and synanthropic animals than in sylvatic hosts. *Trichinella nativa* was detected only in wildlife, and *T. pseudospiralis* was found in four raccoon dogs, one sylvatic wild boar, and one rat. Mixed infections with two *Trichinella* species were detected in five host individuals (5.7% of all positive animals; I, II, III). With multiplex-PCR, raccoon dogs were found to be infected by *T. spiralis*, *T. nativa*, *T. britovi*, and *T. pseudospiralis*, foxes by *T. spiralis* and *T. nativa*, wolves by *T. nativa* and *T. britovi*, cats by *T. spiralis*, rats and wild boars by *T. spiralis* and *T. pseudospiralis*, pigs by *T. spiralis*, and lynxes and bears by *T. nativa* (Figure 4).

Trichinella britovi was detected only in mixed infections, with *T. spiralis* in two raccoon dogs, and with *T. nativa* in one raccoon dog and one wolf. A mixed infection of *T. spiralis* and *T. nativa* was also recorded in one fox (II). In addition to the mixed infections detected by multiplex PCR, there was probably a mixed infection of *T. spiralis* and *T. britovi* in one cat, and of *T. britovi*, *T. nativa*, and *T. spiralis* in one raccoon dog and one wolf according to RAPD-PCR (III). *Trichinella pseudospiralis* was never identified in a mixed infection (II, III).

The presence of *Trichinella* species was partly dependent on host species, with distribution varying between domestic and sylvatic hosts ($p<0.001$). The raccoon dog was the only host infected by all four *Trichinella* species.

Parasite species distribution did not differ significantly between host species in the sylvatic group, nor in the domestic group. However, when the raccoon dog was compared with other sylvatic hosts as a group, the distribution of parasite species was different.

In multiplex PCR, the median number of analyzed larvae per host was 6 (range 1-9). In mixed infections, the corresponding number was 4 (range 3-6), and in single infections 6 (range 1-9).

The relationship between infection intensity and *Trichinella* species was evaluated in the raccoon dog. No significant differences were observed among species (*T. spiralis* average intensity 66 lpg, *T. nativa* 77 lpg, *T. pseudospiralis* 84 lpg) and mixed infections (average intensity 8 lpg; $p=0.38$). Interestingly, the lowest mean intensity was detected in mixed infections.

The results of the outbreak study (I) are consistent with those of the survey on detected *Trichinella* species in different hosts (II, III). Domestic and synanthropic hosts were infect-

ed predominantly by *T. spiralis* (47/49) (+mixed). In sylvatic hosts, the most prevalent species was *T. nativa* (40/57) (+mixed). *Trichinella spiralis* was found in some sylvatic hosts as monoinfections (7/57) or mixed infections (4/57 + 1 observation on wolf by RAPD-PCR).

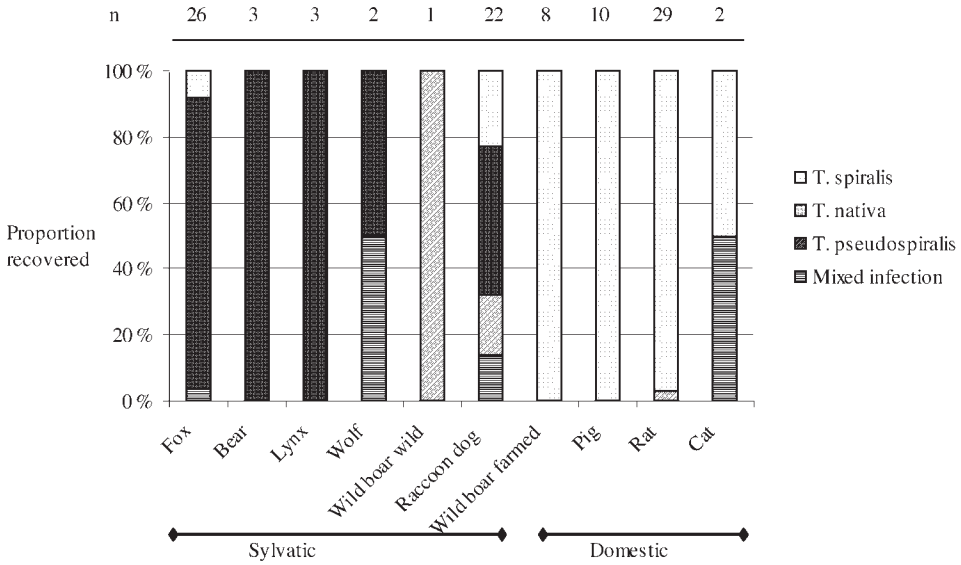

Figure 4. Relative distribution of recovered *Trichinella* species by host species in the sylvatic and domestic cycles (I, II, III). n= number of animals studied.

8.2 Comparison of molecular techniques and other observations

Comparison of the molecular techniques (III) was done by analyzing separate larval samples from the same hosts in two laboratories. PCR-based analyses were carried out on 625 single muscle larvae; 436 were identified by multiplex PCR (II, III) and 189 by RAPD-PCR (III). The total number of analyzed larvae per host ranged from 2 to 12 (median 8). The number of analyzed larvae varied according to availability.

An overall agreement for species identification of *Trichinella* larvae was obtained with the two methods ($K=0.691$, $p<0.001$). However, RAPD-PCR was far more sensitive to the physical condition of *Trichinella* larvae (Fisher's exact test, $p<0.001$) (Table 4). Larvae judged to be in poor condition (uncoiled and/or transparent) yielded smeared, weak bands or no bands with RAPD-PCR. Multiplex PCR produced bands easy to interpret for both molecular weight and similarity to reference strains.

Because *T. pseudospiralis* was not expected to be found in Finland, a reference strain was not included in RAPD-PCR. *Trichinella psudospiralis* findings in four raccoon dogs and one wild boar were therefore interpreted based on experience of the laboratory staff (III).

Simulation of mixed infections showed in RAPD-PCR that *T. nativa* out-competed *T. spiralis*. It yielded a stronger specific pattern in the gel, prevailing over the pattern of *T. spiralis* even when the ratio of *T. spiralis* to *T. nativa* was 2:1 (Figure 5) (I).

Table 4. Number of hosts infected with *Trichinella* sp. according to RAPD-PCR and Multiplex PCR methods (III).

		Multiplex PCR								
		T1	T2	T3	T4	T1+T3	T1+T2	T2+T3	unclear	**Total**
RAPD-PCR	T1	34				1				**35**
	T2		28							**28**
	T3			0						**0**
	T4				5					**5**
	T1+T3	1				1				**2**
	T1+T2						1	2		**3**
	T2+T3									**0**
	unclear	13			1					**14**
	Total	**48**	**28**	**0**	**6**	**2**	**1**	**2**	**0**	**87**

K=0.691 (SE 0.064) p<0.001 (when "unclears" are included)
Proportion of matches (agreements) 69/87 = 0.793
Proportion of matches expected by chance = 0.330

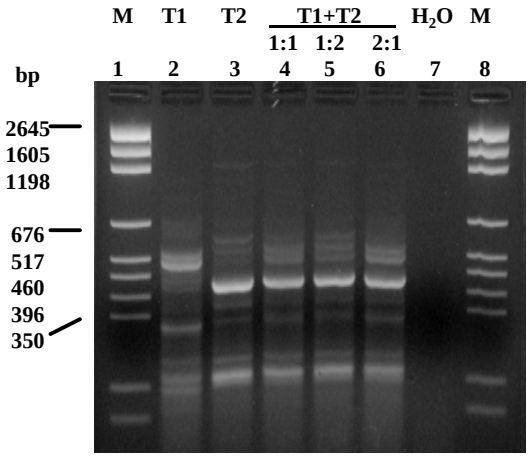

Figure 5. RAPD-PCR gel indicating DNA patterns of samples with a mixture of *T. spiralis* and *T. nativa* larvae. Lanes 1 and 8: PGEM® as a DNA marker, molecular weights on the left. Lanes 2-6: DNA samples of larvae from *T. spiralis* and *T. nativa* reference strains and mixtures in ratios of 1:1, 1:2, and 2:1 in lanes 4, 5, and 6, respectively.

8.3 Experimental studies

8.3.1 Reindeer infection

Inoculated reindeer showed no clinical signs of trichinellosis (IV). The total leukocyte counts were 4-6 × 10^9/l during the study period. No differences were detected relative to infection dose or inoculated *Trichinella* species. The highest leukocyte count among noninoculated animals, i.e. 11.6 × 10^9/l on day 55 post-inoculation, was seen in animal no. 10.

The number of peripheral eosinophilic granulocytes increased slightly during the trial both in inoculated animals and in controls. Relatively high eosinophilic counts were seen in the two *T. nativa*-inoculated reindeer in which no muscle larvae were found. Eosinophilic count tended to be lower in control animals than in inoculated animals.

All inoculated animals seroconverted. An increase in OD in ELISA was seen from day 23 post-inoculation onwards. The OD value was highest around days 30 and 40 in the *T. spiralis*-inoculated group as well as in those inoculated with either 5000 or 15 000 larvae of *T. nativa*. The OD values from the reindeer inoculated with the smallest dose of *T. nativa* (2500 larvae) continued increasing until the end of the study period. Three control animals retained low OD values throughout the trial.

A difference was present in the infection density acquired between the two parasite species (Table 5). The infection density of *T. spiralis* in the pooled samples was 1-2 lpg, whereas that of *T. nativa* remained below 0.1 lpg, regardless of inoculation dose. The masticatory muscles were most heavily infected, but differences between muscle groups were small. Two *T. nativa*-inoculated reindeer were negative by muscle tissue examination of pooled samples.

Table 5. Infection density of *Trichinella* larvae per gram muscle (lpg) in experimentally infected reindeer calves.

Animal number	*Trichinella* species	Inoculation dose, number of larvae	Digestion, pooled sample*	RCI†
1	*T. nativa*	2500	0.04	0.16
2	*T. nativa*	2500	0	<0.04
3	*T. nativa*	5000	0.06	0.12
4	*T. nativa*	15 000	0	<0.007
5	*T. spiralis*	5000	1.96	4
6	*T. spiralis*	5000	1.56	3

* Diaphragm, tongue, and masseter muscle.
† Reproductive capacity index (RCI) (estimated): the total number of muscle larvae established/number of larvae in inoculated dose. The estimate of number of larvae established is based on pooled samples and an estimated muscle mass of 10 kg.

8.3.2 *Trichinella* in feeds

In the experimental study of *T. spralis* persistence in animal feeds (V), some larvae were found in rat carcasses in all tested environments at four weeks of incubation. After six weeks in the shaded box, the muscles of the carcasses were totally decayed, and no *Trichinella* were recovered. In all other environments, small remnants of tissue with identifiable *Trichinella* were found even after six weeks of incubation.

Recovery of *Trichinella* after one-week of incubation in all environments was sufficient to infect four donor rats with 300 larvae apiece. Later, the number of donor rats and the size of the infection dose were chosen based on recovery. After two weeks of incubation, larvae recovered from all environments were infective, but after four weeks only parasites from propionic acid-fermented feed could infect recipient rats. After six weeks of incubation, no parasites were infective (Figure 6).

Rat carcasses were badly decomposed after incubation for six weeks. In silage, only bones and hairs remained of the carcasses. In grained barley, the rat carcasses were mummified, and in propionic acid-fermented feed, large moldy feed clumps surrounded them. In the shaded box, maggots and fur beetles had consumed the carcasses by six weeks. Maggots were found even in one-week samples. The moisture had increased in the carcasses incubated in silage, but target rat carcasses in other environments were desiccated. The pH in all incubated carcasses was higher than in a fresh minced rat carcass.

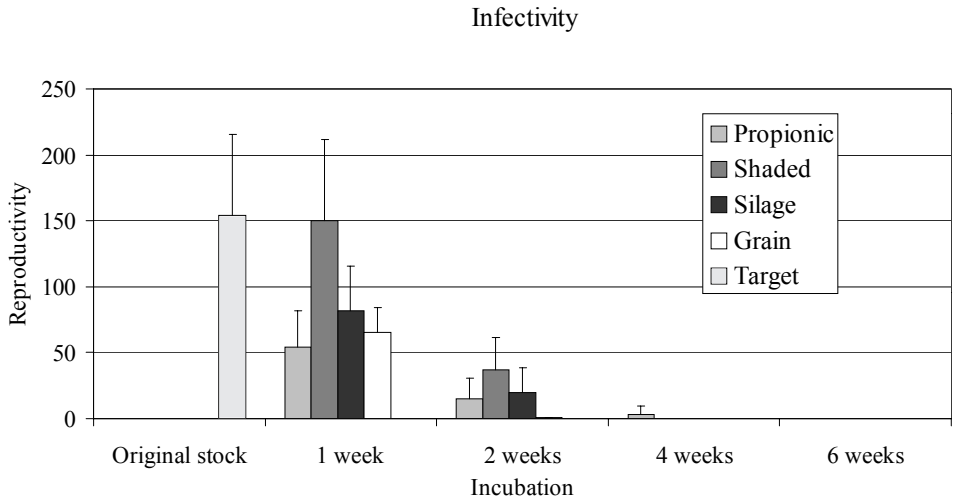

Figure 6. Average reproduction capacity index (RCI) with standard deviation of *T. spiralis* with different experimental feeds and conditions after incubation. Grained barley = “grain”, propionic acid-fermented feed = “propionic”, shaded box = “shaded”.

9. DISCUSSION

9.1 Outbreak on a wild boar farm

In Study I, the rat invasion seemed to be a risk factor for the *Trichinella* outbreak on the wild boar farm. Wild boars that lived during the invasion became infected more often than those born afterwards. Unfortunately, by the time the outbreak of the wild boars was revealed, samples from the rats could no longer be obtained. The prevalence of *Trichinella* infection in foxes around the farm was generally even higher than in foxes from southeast Finland (II). However, the dominant parasite species in foxes was different from that in wild boars, suggesting that the foxes might not be the source of the infections in wild boars. On the other hand, it is unclear, whether the foxes had a mixed *Trichinella* infection since *T. nativa* dominates *T. spiralis* in RAPD-PCR results.

Other studies have demonstrated that rats can maintain a *Trichinella* infection on a pig farm. Moreover, wildlife living around a pig farm can be infected by the same parasitic strain as the pigs. The infection had probably spread from the farm to the surroundings rather than *vice versa* (Murrell *et al*., 1987; Schad *et al*., 1987; Leiby *et al*., 1988). In Finland, rats from dumps have been shown to often carry *Trichinella* infection (Mikkonen *et al*., 2005).

9.2 Risk of infection by regions and hosts

According to our results (II), the prevalence of infection in red foxes and raccoon dogs has increased as compared with reports from the 1950s to the 1980s (see the Review of Literature). The prevalence among lynxes in southern regions of Finland was also higher than that found in earlier studies, but the geographical variation in prevalence is in accordance with previous data (Oksanen *et al*., 1998).

Sensitivity in detecting infection in a host depends on the muscle sampled and size of the sample. The examination for larval burden is more sensitive when the sampling is focused on known predilection sites of a particular host species (Serrano *et al*., 1999; Gamble *et al*., 2000; Nöckler *et al*., 2000; SCVPH, 2001). There was some variation in sampling sites in Study II. This may have thus caused biases in the results, which must be considered when comparing data of different host species. However, within a host species, sampling sites were fairly constant. The samples were taken from muscles known to be predilection sites in carnivores (Kapel *et al*., 1994; Kapel *et al*., 1995; Mikkonen *et al*., 2001).

In bears, the screening for trichinellosis was done through meat inspection. The technique used was either artificial digestion or trichinoscopy, but it was not recorded. The sample size of 1.0 g or only 0.2-0.3 g per animal, respectively, had a clear effect on the sensitivity of the screening. Probably more than 50% of the bears were examined with trichinoscopy. Undoubtedly, the recorded prevalence in bears was lower than it would have been with the 1.0-g samples used in the digestion method. The variation in all examined sample sizes of differ-

ent host species and the larvae being distributed unevenly in the muscles suggest that some low-intensity infections were probably also reported.

9.3 *Trichinella* species detected in different hosts

No significant difference was present in the distribution of *Trichinella* species among host species in the southwest and southeast regions of Finland (II), but the number of examined animals per host species was small and the hosts did not originate from the two regions in equal numbers. Five of six animals harboring *T. pseudospiralis* originated from wildlife in the southeast region. In contrast, most domestic, synanthropic, and sylvatic animals infected with *T. spiralis* were from southwest Finland. The sampled hosts were all heavily infected in the parasite species identification part of the Study II, unlike in the prevalence part of the same study. The most intensively infected hosts were chosen for this study to enable sufficient numbers of larvae to be collected from each sample. Since different *Trichinella* species breed with different efficiency in different host species, there might be a bias in the findings, with the most effectively breeding *Trichinella* species dominating. According to Pawlowski (1981), a low-intensity infection in pigs results either from an infection with *Trichinella* species not specific to pigs or from an infective dose below 50 larvae per pig. If *Trichinella* species were analyzed only in intense infections, biased results could occur.

All larvae identified at the species level originated from animals sampled in southern Finland. However, the literature on geographical and host species-specific distribution of *Trichinella* species indicates that the most likely etiologic agent of sylvatic trichinellosis in northern Finland is the freezing-resistant species *T. nativa* (Shaikenov and Boev, 1983; La Rosa *et al*., 1992; Pozio *et al*., 1992a; Pozio *et al*., 1998; Pozio, 2001).

An unpublished observation of a freezing-resistant *Trichinella* sp. has also been made in a bear from northern Finland (Oivanen, 1987 unpublished). The host animal originated from Pelkosenniemi, Lapland. After freezing the muscles for five days at –18°C, two Swiss mice were infected with a 1-g 43-lpg dose of the meat, resulting in infections of 77 lpg and 7 lpg in the mice.

Sylvatic species *T. britovi* and *T. nativa* were not detected in pigs, although a high prevalence of trichinellosis does exist in wildlife, with the predominant species being *T. nativa*. Circumstances in nature probably favor *T. nativa* because of its high resistance to freezing in naturally infected carnivores (Kapel *et al*., 1999) in the subarctic climate of Finland. This species shows a very low RCI in swine and rodents (Pozio *et al*., 1992a; Kapel and Gamble, 2000; Kapel, 2001). To date, only two reports have been published of a natural *T. nativa* infection in pigs, one in a domestic pig (Gasser *et al*., 1998) and the other in two sylvatic wild boars (Pozio and Kapel, 1999). The risk of transmission of *T. nativa* from wildlife to the domestic habitat appears to be rather small. However, an observation has been made of a pig-derived Finnish *Trichinella* isolate that infected Swiss mice after freezing the pork in small pieces for 24 h at –18°C (Hovi and Oivanen, 1985). Unfortunately, at that time, species determination was not yet available.

Trichinella britovi was detected in Finland for the first time. The finding was expected because the species occurs in neighboring countries Sweden and Estonia (Christensson, 1994; Pozio *et al*., 1995).

Trichinella pseudospiralis was also detected in Finland for the first time in this study. The several findings of *T. pseudospiralis* were unexpected since the species had not been detected in northern Europe before. Because the species is able to infect birds, its transmission potential is enormous. These findings together with recent reports from Sweden support the assumption that this species occurs widely in the northern areas of the northern hemisphere (Christensson and Pozio, 2004; Pozio *et al*., 2004b).

Rats, cats, foxes, raccoon dogs, and wild boars were infected with the domestic species *T. spiralis*. *Trichinella spiralis* was shown to occur in wildlife in Finland. However, whether *T. spiralis* is maintained in the sylvatic cycle and transmitted back to the domestic environment is unknown. The absence of sylvatic *Trichinella* in domestic pigs might be explained by the very low infectivity of *T. nativa, T. britovi,* and *T. pseudospiralis* for pigs and wild boars (Murrell *et al*., 1986; Kapel *et al*., 1998; Kapel and Gamble, 2000; Kapel, 2000; 2001). However, natural infection occurs under certain circumstances. *Trichinella britovi* has been detected in a domestic pig near Finland (Pozio *et al*., 1995; Järvis *et al*., 2002). *Trichinella nativa* and *T. britovi* have low infectivity also for rats (Pozio *et al*., 1992a; Malakauskas *et al*., 2001), which weakens the probability of rats carrying the infection into piggeries.

9.4 Mixed infections of several *Trichinella* species

The median intensity of infection was higher in the host animals utilized for species detection than in those in the prevalence study. If some *Trichinella* species bred more efficiently in certain host species, then these parasite species would dominate in the results of mixed infections.

The minimum number of larvae needed for analysis of possible mixed infection can be roughly estimated by binomial distribution. Theoretically, supposing that two or more equally prevalent species occurred in equal proportions, the number of larvae needed per host to find at least two species with a probability of 0.95 is 6. However, if larvae are not distributed equally, the minimum number of larvae required increases markedly. The present results and literature suggest that mixed infections can be detected when a sufficient quantity of animals is examined in an area where two or more species of *Trichinella* coexist (Pozio *et al*., 1997; Pozio *et al*., 1998; Pozio, 2000). Coincidentally, the number of studied larvae in single-species infections was higher than the number in mixed infections (II, III). This might indicate that in most cases there were a sufficient number of observations for detection of a mixed infection.

Concurrent infections have previously been described with *T. britovi* and *T. nativa* in raccoon dogs and foxes (Pozio *et al*., 1995; Pozio *et al*., 1998), and with *T. spiralis* and *T. britovi* in wild boars (Pozio *et al*., 1997). The mixed infection with *T. spiralis* and *T. nativa* reported here has also been found in Sweden (Pozio, 2000; Pozio *et al*., 2004b). The occurrence of

mixed natural infections indicates that immunity from an initial infection may not protect from all re-infections, at least not from other species.

9.5 Comparison of molecular techniques

Comparison of the results yielded by multiplex PCR and RAPD-PCR (III) shows that the former offers a clear advantage. Multiplex PCR was much less influenced by suboptimal preservation of parasite DNA. This difference was most apparent in the 14 host animals for which RAPD-PCR was negative for any *Trichinella* sp. while multiplex PCR was positive. For mixed infections, with the exception of three results, RAPD-PCR gave similar results to multiplex method. The multiplex PCR agarose gels were easier to read than the RAPD-PCR gels due to their simple and distinct band pattern. Thus, the results of multiplex PCR were less subjective. Costs of the two techniques are approximately equal, as is the laboriousness. Both methods need to be optimalized and standardized when taken into use in other laboratories.

The following factors can explain the discrepancy in results between RAPD-PCR and multiplex PCR when mixed infections were analyzed: 1) RAPD-PCR and multiplex PCR analyze different parts of genomial DNA, 2) the RAPD-PCR method is very sensitive to deteriorated DNA, and 3) the DNA analyzed by the two techniques was actually different. While the larvae originated from the same host individuals, the DNA was extracted from different larvae. This could account for some samples giving *T. spiralis* or *T. britovi* results in RAPD-PCR and a conflicting result in multiplex PCR. Since we know that mixed infections exist, the divergent results could both be correct.

Among the farmed wild boars and the foxes captured around the farm, no signs of mixed infections of different *Trichinella* species were detected (I). However, the larval DNA was most often extracted from pooled samples of 10 larvae. The testing of artificial samples of mixed *Trichinella* species showed that *T. nativa* predominated over the bands of *T. spiralis* in RAPD-PCR gel. The detection of *T. spiralis* in pooled larval samples from the wild boars and in one of the foxes therefore likely represents a genuine monoinfection. In most of the foxes, the dominant *T. nativa* parasite was detected but possible mixed infections may simply have been underdiagnosed.

In detecting parasite species in mixed infections, multiplex PCR seems to be more useful than RAPD-PCR. Experimentally mixed DNA samples analyzed by reverse line hybridization have also yielded clear results (Rombout *et al*., 2001).

The correlation between results of RAPD-PCR and multiplex PCR is high under ideal conditions, but multiplex PCR also permits identification of material that has been frozen and thawed repeatedly and subsequently preserved in ethanol. This technique is therefore more suitable for epidemiological studies.

9.6 Serological findings compared with microscopic results

In wild boar and reindeer, ELISA more often showed positive test results than the digestion method. One of three wild boars with immunoreactive *Trichinella* antibodies was negative at meat inspection but had the highest absorbance in ELISA (I). All reindeer inoculated with *T. nativa* seroconverted, but only two could be parasitologically confirmed to be infected (IV).

Serological methods have been found to be more sensitive than direct microscopy in detecting *Trichinella* infections (Kapel *et al*., 1998; Yepez-Mulia *et al*., 1999; Nöckler *et al*., 2000; Sukura *et al*., 2001). The difference between serological and digestion results of reindeer inoculated with *T. spiralis* and *T. nativa* may be due to a differing ability of the parasite species to preserve in the host tissues. This may depend on characters of both the parasite species and the host (Wakelin and Goyal, 1996; Kapel, 2001; Wakelin *et al*., 2002; Bolas-Fernández, 2003). In reindeer, the highest larval burden was apparently caused by *T. spiralis*. The serological response of the wild boar negative at meat inspection could have been due to infection with *T. nativa* or *T. pseudospiralis*. On the other hand, serological methods are known to show higher prevalence than direct methods because of lower specificity due to cross-reactions or poor quality of blood samples. The pre-muscle stage of infection could also be detected as positive with ELISA but negative with digestion. ELISA is not recommended for individual carcass inspection (Gamble *et al*., 2004).

9.7 Experimental infection of reindeer

In Study IV, the arctic species *T. nativa* was less infective for reindeer than the domestic species *T. spiralis*. The distribution of muscle larvae in reindeer was roughly similar to that demonstrated in other ruminants (Alkarmi *et al*., 1990; Smith *et al*., 1990; Tomašovièová *et al*., 1991; Reina *et al*., 1994; Theodoropoulos *et al*., 2000; Moretti *et al*., 2001). The masticatory muscles and the tongue had the highest larval burden, but the differences between muscles were small. Even the most infected animals (with *T. spiralis*) harbored only a low number of parasites. The estimated RCI for *T. spiralis* in reindeer was thus only 3 or 4, whereas in Wistar rats an RCI of about 200 has been reported by Pozio *et al*. (1992a). In our study (V), the mean RCI for *T. spiralis* in Wistar rats was 150 ($\pm$ 60 SD). For *T. nativa*, the RCI in reindeer was low, <0.2. This corresponds to observations in sheep and cattle (Smith and Snowdon, 1989; Smith *et al*., 1990; Theodoropoulos *et al*., 2000). In swine, *T. nativa* either produced no muscle larvae (Kapel *et al*., 1998) or produced very few (Kapel and Gamble, 2000), and in rats the RCI was about 0.1 (Pozio *et al*., 1992a). More suitable hosts for *T. nativa* are, for example, laboratory mice, with an RCI of 14-15 (Bolas-Fernández and Wakelin, 1989; Webster *et al*., 1999) and guinea pigs, with an RCI of 420 (Webster *et al*., 1999). In the Swiss mice used for the inoculum in this trial (IV), the *T. nativa* RCI was as high as 75. In some carnivores, detected muscle larvae densities have also been relatively high (up to hundreds of *T. nativa* lpg), like in arctic foxes from Greenland (Kapel *et al*., 1995) and a red fox from Estonia (Pozio *et al*., 1998). Those results indicate successful reproduction in the host. When RCI is below 1, it indicates that the number of the next generation is smaller than the parent generation.

Horses, which represent a herbivorous host species, have been reported to be infected by *Trichinella sp*. in different parts of the world (Boireau *et al*., 2000). Some reports do exist of reindeer and other herbivores in the Arctic voluntarily eating meat (Kokko, 1947; Madsen, 1961). In nature, if reindeer were to eat meat, they would probably swallow rodents more or less whole. In this trial, the experimental animals were inoculated by minced mice carcasses. Compared with consuming intact rodents, minced meat may be a more effective way of passing an infective parasite to the intestine of a new host. In experimental anaerobic digesters, *T. spiralis* larvae were able to survive a maximum of 96 h (Fitzgerald and Prakasam, 1978).

All six inoculated reindeer seroconverted. The seroconversion was seen in the reindeer at 3-4 weeks post-inoculation, which indicates that all animals were truly exposed, including the two that remained parasitologically negative. Even though no larvae were found in the samples from reindeer nos. 2 and 4, they might have harbored small numbers of larvae. In addition, animal nos. 1 and 3 had very low infection densities, which may have led to false-negative results. The OD values of individual animals observed in the *T. nativa*-inoculated group seem to correlate with the inoculated dose of larvae.

Reindeer meat is not traditionally eaten raw but is often eaten dry-cured in Fennoscandia. Nevertheless, the tissue water activity, salt concentration, and curing temperature all have an impact on the persistence of *Trichinella* species in cured pork (Zimmermann, 1971; Smith *et al*., 1989). While dry-cured reindeer meat probably does not possess a high risk of trichinellosis for humans, according to international recommendations, curing and smoking are not reliable for control of *Trichinella* in pork, horse, or game meats (Gamble *et al*., 2000).

9.8 Experiment on *Trichinella* infected feeds

All the different feed processing methods tested decreased the infectivity of *Trichinella* in two weeks. Infectivity of *Trichinella* larvae was maintained best in rat carcasses kept in the shaded box, but because the flesh was devoured by maggots, no larvae could be recovered, and the RCI could not be confirmed beyond four weeks. *Trichinella* larvae can survive and be infective when ingested by maggots. Their survival in maggots depends on environmental temperature, but will not exceed five days (Maroli and Pozio, 2000).

Age of the infection is known to influence the resistance of muscle larvae to decomposition. After 37 weeks of infection, muscle larvae resisted putrefaction better than after five weeks of infection (von Köller *et al*., 2001). The resistance was better in rodent muscles than in carnivore muscles, and of the different parasite species, *T. britovi* and *T. nelsoni* resisted best. In view of the results of von Köller and others, *T. britovi* might be more resistant in feeds than *T. spiralis* was in our study. The persistence might have been even longer than four weeks had the initial infection been older.

In Finland, silage packed in bales is often stored outdoors until used, also in wintertime. Freezing does not spoil the feed. Those *Trichinella* species resistant to freezing may survive in contaminated fodders even during northern European winters. A lower environmental

temperature and/or anaerobic circumstances may prolong the persistence of infectivity in feeds (Stewart *et al*., 1990; Jovic *et al*., 2001). The ability of *Trichinella* to maintain infectivity in different feeds for a few weeks might explain some of the unexpected herbivore hosts known to be sources of human outbreaks (Boireau *et al*., 2000; Pozio, 2001).

In an endemic area, rodents may also increase trichinellosis risk in indoor animals both by contaminating their feed and by being the prey or scavenging source of such animals as pigs (Murrell *et al*., 1987; Schad *et al*., 1987). Fresh hay is given to animals soon after harvesting. In our experiment, infectivity in the pasture-condition simulation was completely unaffected in one week. For this reason, contaminated rat carrion mixed with hay or milled on a farm may well be a source of an outbreak. The typical management practice of milling the grain on the farm and mixing it with protein concentrate does not include long storage. Two weeks' persistence of infectivity can thus be hazardous for farm animals if infected rats have access to the crop storage. Silage is recommended to be fermented for at least one month before use. In summer temperatures in this study, infectivity in silage was minimized by four weeks' incubation. It is worth noting that after four weeks infective larvae were still found in propionic acid-fermented feed. Grain-based and hay-based feeds contaminated with *Trichinella* larvae can easily be a risk for farm animals.

9.9 *Trichinella* cycles in Finland

Annual observations of pig trichinellosis are rare in most western European countries. The epidemiological situation in Finland resembles that of Baltic countries more so than that of western European countries. However, no human cases have been reported in Finland for almost 30 years. Some explanations for the lack of human infections could be extensive meat inspection of domestic and farmed animals, local preference for well done pork, and very little home slaughtering.

Rats have traditionally been assumed to be reponsible for swine trichinellosis in Finland, but the hypothesis has remained difficult to prove. In Croatia, southern Europe, rats seemed to be only accidental hosts of *T. spiralis*, not a reservoir for pigs at farm level (Stojcevic *et al*., 2004). In Finland, although rats from dumps have been demonstrated to often carry trichinellosis (Mikkonen *et al*., 2005), their role in infecting pigs is unknown. The dominating species detected in rats was *T. spiralis*. In the epidemic on the wild boar farm, the indirect evidence suggested the cause to be the earlier rat invasion; however the rats were not available for sampling.

The absence of *T. nativa* in the rat population is most likely explained by its limited infectivity for rats (Pozio *et al*., 1992a; Malakauskas *et al*., 2001). In any case, *T. nativa* is currently also absent from the pig population. The finding of *T. pseudospiralis* in a rat and in both farmed (Sukura *et al*., 2001) and sylvatic wild boars complicates the picture of *Trichinella* cycles in Finland. Furthermore, *T. britovi* was detected in a farm cat (III).

The population density of foxes and raccoon dogs is higher in western than in eastern Finland, but lynxes tend to be more abundant in eastern Finland (Kauhala, 1996a; 1996b;

Kauhala and Helle, 2000). The average populations of foxes and raccoon dogs are roughly similar in size in the spring. The average age of raccoon dogs is probably lower than that of foxes because raccoon dogs have larger litters and are more likely to die during the winter (Kauhala, 1996a; 1996b). The population age may have an influence on *Trichinella* prevalence since older animals have been exposed longer to the infection. The higher prevalence of trichinellosis in wildlife in southern than in northern Finland has been suggested to be related to the role of the raccoon dog as a reservoir for *Trichinella* infection (Oksanen *et al*., 1998). The finding that mixed infections occurred more frequently in raccoon dogs than in other carnivores is in accordance with this species scavenging nature. The mean intensity of the infection in raccoon dogs was unrelated to parasite species, suggesting that all species of *Trichinella* detected were adapted to the raccoon dog. The mean infection intensity in raccoon dogs was lowest with mixed infections, which could represent an indirect expression of partial immunity from earlier infections. However, in other host species, differences have been reported between the breeding capacities of different *Trichinella* species (Pozio *et al*., 1992a; Kapel *et al*., 1998; Webster *et al*., 1999; Kapel, 2001; Malakauskas *et al*., 2001). Experimental studies have concluded that not even a high exposure to *Trichinella* infection results in severe or long-term clinical signs in raccoon dogs (Näreaho *et al*., 2000). The good adaptation of trichinellosis in the raccoon dog and the great numbers of this host in both sylvatic and domestic habitats in Finland (Helle and Kauhala, 1991) support the hypothesis that this mammal could be an important infection reservoir, forming the link between domestic and sylvatic life cycles.

Data on associations between pathogen species and hosts are consistent with the speculation that in Finland *T. spiralis* cycles among host species close to human habitation. However, the other three *Trichinella* species also appear to cycle near human habitation. The traditional classification to domestic and sylvatic cycles may thus not be very useful in Finland. The domestic species *T. spiralis* together with the other three species also seem to cycle in sylvatic hosts. Sometimes the different *Trichinella* species are involved in a mixed infection of a single host. The observations show that a categorical classification of the epidemiology into sylvatic cycle versus domestic cycle is somewhat artificial. In Finland, the sylvatic hosts foxes and raccoon dogs, which have fairly large populations, typically live close to human habitation and feed on, for example, garbage heaps of farms. Even bears and wolves have been observed close to human habitation. Lynxes likely represent the only true sylvatic host species.

10. CONCLUSIONS

10.1 Domestic and sylvatic hosts of *Trichinella* infection

In Finland, trichinellosis is much more common in farmed wild boars than in domestic pigs. These host animals of the same species (*S. scrofa)* are raised differently; pigs are generally kept indoors, while farmed wild boars are fenced outdoors. The influence of the environment *i.e.* the high prevalence in wildlife is thus seen by comparing the prevalence in farmed to sylvatic wild boars.

An example of environmental influence can be seen in the outbreak of trichinellosis at the wild boar farm, where a rat invasion seemed to be a risk factor for the infection. The prevalence of *Trichinella* infection in foxes around the farm was even higher than in foxes from southeast Finland, revealing that local conditions favored *Trichinella* infections.

Semidomesticated reindeer raised traditionally in north Finland were shown to be experimentally infected with *Trichinella*. The arctic species *T. nativa* was less infective for reindeer than the domestic species *T. spiralis*. These half-tamed ruminants may act as hosts for *Trichinella*.

The observed prevalence of trichinellosis in wildlife varied widely according to host species and region of the country. The prevalence in bears and foxes was low in the north and high in southern part of the country. In the southwest, the highest prevalence was observed in lynxes, whereas in the southeast, the highest rate was found in foxes. The prevalence of infection in foxes has increased as compared with reports from earlier decades. The highest infection intensity was detected in raccoon dogs.

10.2 *Trichinella* species in different host species

Of the four *Trichinella* species detected in Finland, *T. britovi* and *T. pseudospiralis* were detected for the first time. *Trichinella pseudospiralis* had not been detected in northern Europe before the present work. The dominant *Trichinella* species in domestic hosts in Finland is *T. spiralis*, which is the classical species in domestic cycle. In sylvatic hosts, the most prevalent *Trichinella* species is *T. nativa*. *Trichinella nativa* has not yet been detected in domestic hosts, despite a strong infection pressure from wildlife. All of the four *Trichinella* species were detected in Finnish wildlife in the sylvatic cycle.

Mixed infections of different *Trichinella* species were detected in several hosts. In the raccoon dogs, the proportion of mixed infections was biggest among sylvatic hosts. Most of the infections of *T. pseudospiralis* were detected in raccoon dogs. In addition, it was the only host infected by all four *Trichinella* species.

Comparison between RAPD-PCR and multiplex PCR methods revealed that multiplex PCR is more useful for identification of *Trichinella* species in epidemiological studies. Multiplex PCR tolerates suboptimal DNA quality better than RAPD-PCR, and interpretation of gel results is less ambiguous.

10.3 Epidemiological situation in Finland

Grain- and hay-based feeds contaminated with *Trichinella* larvae can easily carry the infection to farm animals. In addition, rats may be a likely vector of pig infections, although their role remains unconfirmed. Raccoon dogs and foxes presumably maintain the infection pressure close to farms.

The domestic species *T. spiralis* together with the other three species seem to cycle in sylvatic hosts relatively close to human habitation. The traditional classification to domestic and sylvatic cycles may therefore be of limited use in Finland.

The raccoon dog differed from the other sylvatic hosts analyzed since it served as a host for all four *Trichinella* species, carried the most intense infections, and had the largest proportion of mixed infections. This host species may be an incubator of *Trichinella* parasites in Finnish wildlife.

Special characteristics of the epidemiological situation in Finland include sporadic domestic pig trichinellosis (*T. spiralis*), presence of four *Trichinella* species in the same regions, presence of *T. spiralis* in sylvatic wildlife, presence of *T. pseudospiralis* in many host species, and mixed infections of several *Trichinella* species.

11. ACKNOWLEDGMENTS

The present studies were mainly carried out at the Section of Veterinary Pathology and Parasitology, Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki. Some parts of the project were done at the Department of Food Hygiene, National Veterinary and Food Research Institute, Helsinki. I was also fortunate to have the opportunity to work at the Danish Centre for Experimental Parasitology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark. The Zoological Gardens, University of Oulu, the Oulu Regional Unit, National Veterinary and Food Research Institute, Oulu, and the International *Trichinella* Reference Centre, Istituto Superiore di Sanità, Rome, Italy, are thanked for co-operation.

I extend my warmest thanks to my colleagues at these institutes and all others who contributed in one way or another. This has been a very interesting and educative project for me, and many of you have become my good friends over the years.

I am especially grateful to:

- Professor Antti Sukura, DVM, PhD, Head of the Department of Basic Veterinary Sciences and my supervisor, for his never-ending enthusiasm for scientific studies and for his support, advice, and friendship during the years we have worked together.
- Professor Tuula Honkanen-Buzalski, DVM, PhD, Director General of the National Veterinary and Food Research Institute and my supervisor, for her support and encouragement and for placing the facilities of the institute at my disposal.
- Docent Jorma Hirn, DVM, PhD, for introducing me to the fascinating subject of *Trichinella* epidemiology when I was still a student looking for a topic for my advanced studies, as well as being supportive and providing sound advice.
- Osmo Mäki-Petäys, DVM, Director of the Meat and Fish Hygiene Unit, National Food Agency, for his encouraging attitude towards my studies and for the understanding I got on my peculiar interest.
- My research colleagues, members of the *Trichinella* team: Taina Mikkonen, MSc, and Anu Näreaho, DVM, for warm friendship, a good sense of humour, fruitful collaboration, and empathy during this arduous process.
- My coauthors: Professor Christian Kapel, Danish Centre for Experimental Parasitology, Royal Veterinary and Agricultural University, Frederiksberg, Denmark; Professor Edoardo Pozio and Dr Giuseppe La Rosa, the International *Trichinella* Reference Centre, Istituto Superiore di Sanità, Rome, Italy; Docent Antti Oksanen, DVM, PhD, Oulu Regional Unit, National Veterinary and Food Research Institute, Oulu, Finland; Kjetill Åsbakk, PhD, and Taneli Tirkkonen, DVM, The Norwegian School of Veterinary Science, Department of Arctic Veterinary Medicine, Tromsø, Norway; Eija Elomaa, MD, Department of Physiology, University of Oulu, Oulu, Finland; Heli Karhula, DVM, Department of Basic Veterinary Sciences, and Laura Haltia, DVM, and Professor Hannu Saloniemi, DVM, PhD, Department of Clinical Veterinary Sciences, Faculty of Veterinary Medicine, University of Helsinki.

- Alvin Gajadhar, DVM, PhD, and Docent Riitta Maijala, DVM, PhD, the official reviewers of my dissertation, for constructive criticism and suggestions that greatly improved the text.
- Carol Ann Pelli, HonBSc, for language editing and comments on the manuscript.
- Teresa Skrzypczak, DVM, for help with some articles written in Russian and Anna-Maija Grönlund, DVM, and Maaria Paananen, DVM, for help with articles written in French.

Financial support from the Emil Aaltonen Foundation, the Research Foundation of Veterinary Sciences, the Finnish Veterinary Science Foundation, the Farmos Research and Science Foundation, the Walter Ehrström Foundation, and the University of Helsinki is gratefully acknowledged.

Finally, my heartfelt thanks are due to my family: my children Sini and Samuli for being the joy and pride of my life, and my mother Terttu for enormous help and for having unquestioning faith in me. Without this support, I would never have gotten this work done.

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